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Carotenogenic Gene Expression and Carotenoid Accumulation in Three Varieties of *Cucurbita pepo* during Fruit Development

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Supporting Information

ABSTRACT: The control of gene expression is a crucial regulatory mechanism in carotenoid accumulation of fruits and flowers. We investigated the role of transcriptional regulation of nine genes involved in the carotenoid biosynthesis pathway in three varieties of *Cucurbita pepo* with evident differences in fruit color. The transcriptional levels of the key genes involved in the carotenoid biosynthesis were higher in flower-, leaf-, and fruit skin tissues than flesh tissues. This correlated with higher concentration of carotenoid content in these tissues. The differential expression among the colored and white cultivars detected for some genes, such as *LCYe*, in combination with other regulatory mechanisms, could explain the large differences found in terms of carotenoid content among the three varieties. These results are a first step to elucidate carotenogenesis in *C. pepo* and demonstrate that, in general, regulation of the pathway genes is a critical factor that determines the accumulation of these compounds.

KEYWORDS: *carotenoid, Cucurbita pepo, lycopene ε-cyclase, lutein, transcriptional regulation*

INTRODUCTION

Carotenoids are naturally occurring pigments widely present in organisms as diverse as fish, invertebrates, birds, and even algae, bacteria, and fungi.[1] In plants, they are responsible for the external and internal coloration of organs such as fruits, roots, and flowers and are synthesized in the plastids. In chloroplasts of green tissues, carotenoids are known to function as light-harvesting pigments and are essential for protecting tissues against photo-oxidative damage.[2] Nevertheless, in chromoplasts of fruit and flowers, they can be considered as secondary metabolites having an important role to provide different colors to tissues in order to attract pollinators and seed dispersers.[3,4] The health benefits of carotenoids are related to their antioxidant activity and involvement in removing reactive oxygen species, which may cause oxidative damage to cells.[5] They have a high potential for alleviating age-related diseases, and in addition, some carotenoids such as *β*-carotene are precursors of vitamin A and therefore essential for normal vision.[6]

Carotenoids cannot be synthesized by humans and must be acquired through diet. Carotenoid engineering in crops is a potential tool to increase their content in the diet,[7,8] hence it is important to understand the molecular mechanisms regulating carotenoid biosynthesis. In this regard, many advances on genetic manipulation have been achieved in several crops such as tomato, rice, and potato.[9−11]

Carotenoids are 40-carbon tetraterpenes built from eight 5-carbon isoprenoid units joined in such a way that the order is reversed at the center of the molecule.[12] Hydrocarbon carotenoids are collectively termed carotenes: those containing oxygen are called xanthophylls. In plants, carotenoids are synthesized in the plastids, produced via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway.[13] The first steps in the MEP pathway are regulated by 1-deoxyxylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), while the last committed step is regulated by 1- hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (HDR). This metabolic pathway produces the substrate for the carotenoid biosynthesis pathway and has been reported to influence carotenoid content.[14,15] In the carotenoid biosynthetic pathway, the first committed step is the condensation of two molecules of geranyl geranyl pyrophosphate (GGPP) to form phytoene, catalyzed by the enzyme phytoene synthase (PSY). Desaturation and isomerization of phytoene occurring in higher plants is catalyzed by phytoene desaturase (PDS), *ζ*-carotene desaturase (ZDS) and two isomerases, *ζ*-carotene isomerase (ZISO) and carotene isomerase (CRTISO), leading to the formation of lycopene. At this step the carotenoid pathway branches at the cyclization of lycopene. The lycopene *β*-cyclase (LCYb) catalyzes the formation of *β*-carotene introducing two *β*-rings into lycopene, while lycopene *ε*-cyclase (LCYe) can only incorporate one *ε*-ring and needs LCYb activity to form *α*-carotene.[16,17] *α*-Carotene is converted in two steps to lutein, while *β*-carotene, after two hydroxylation steps, is converted to zeaxanthin.[18,19]

Cucurbita pepo L. is a monoecious plant divided within eight morphotypes grouped into two subspecies: subsp. *pepo* L.

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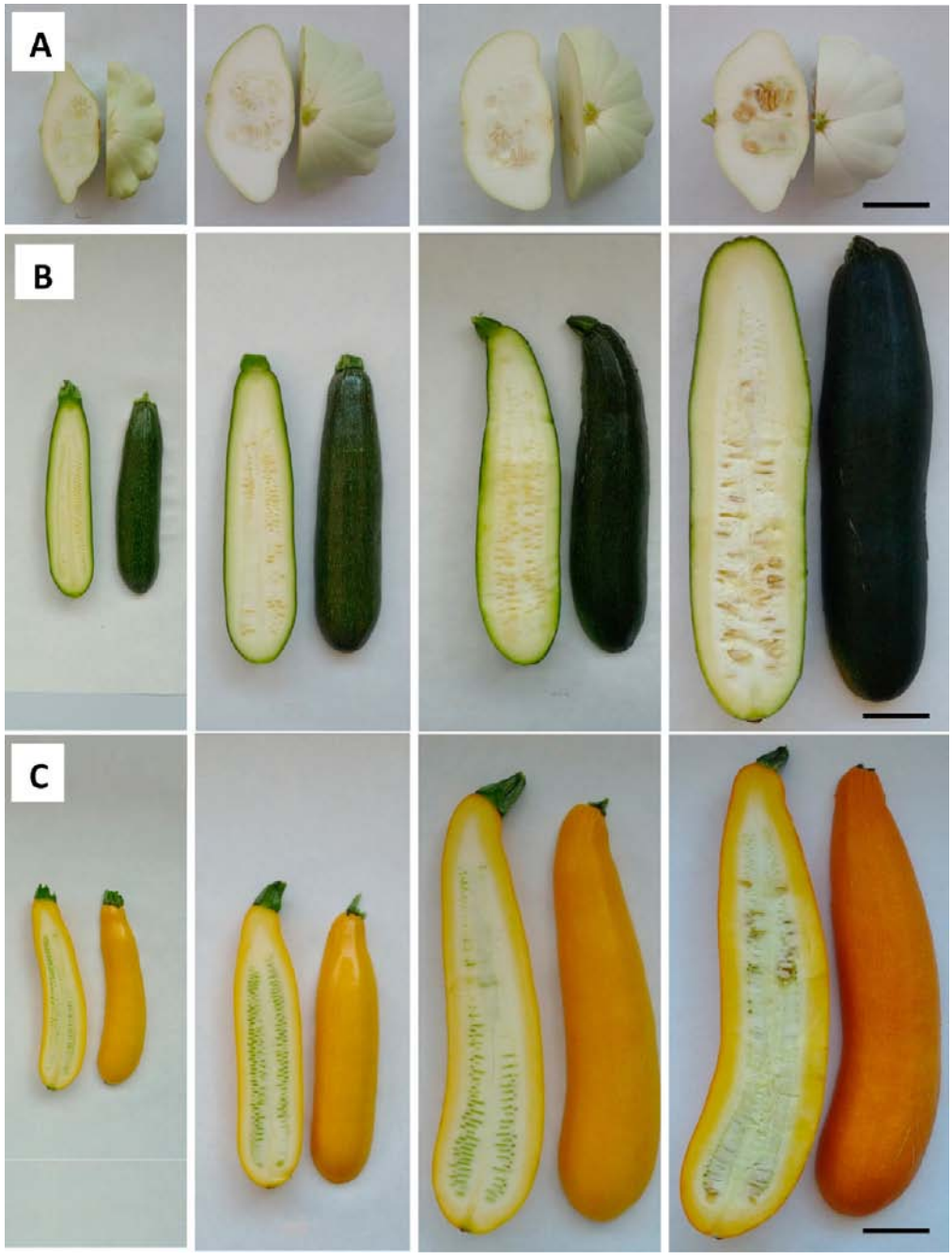

Figure 1. *Curcubita pepo* varieties during fruit development: (A) Scallop, (B) MU_CU16, (C) Parador. Scale bars represent 5 cm.

(pumpkin, vegetable marrow, cocozelle, and zucchini) and subsp. *ovifera*/*texana* L. (scallop, acorn, crookneck, and straightneck).[20] *C. pepo* is one of the most variable genera within the Cucurbitaceae family and is the species with the greatest monetary value of the genus.[21,22] This polymorphism is particularly evident in fruit: size, shape, texture, and color. As in many fruits or vegetable, carotenoids in *C. pepo* contribute to a wide range of colors and concentrations of these substances vary among species and cultivars. Although the green skin variety is the most commercial and well-known, *C. pepo* shows a considerable variation in fruit color such as green, yellow, and orange, ranging in intensity and shading from almost black to almost white. Moreover, accumulation of carotenoid in *C. pepo* during fruit development can lead to changes in pigmentation.[23] Studies of the regulation of carotenoid accumulation during fruit development in plants have been carried out, showing that the regulation of transcriptional level of carotenogenic genes is principal factor controlling carotenoid accumulation.[14,24−27]

To date, although carotenoid accumulation and content have been studied in *C. pepo*[24,28] the transcriptional analysis of the biosynthetic pathway genes has been only reported in winter squashes: *C. maxima*, *C. moschata*, and two lines of their interspecific inbred lines.[29] In this study, we have studied carotenogenic gene expression profiles in three *C. pepo* genotypes with different fruit peel color: white, green, and yellow−orange. Two of these genotypes, zucchini (ssp. *pepo*) and scallop (ssp. *ovifera*) (green and white peel color, respectively) are particularly of great interest because they are the parental lines of the segregating population used to

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Table 1. Primers Used for Cloning cDNA

gene	forward and reverse primers 5′→ 3′	T_m (°C)	amplicon size (bp)
PSY	F: GTT GGG TTG ATG AGT GTY CCT R: GAA GTT TCT CCA TTT ATC AGT	50	241
PDS	F: GAG GTB TTY ATT GCH ATG TCM AAG GC R: TGR TCH GCW GMR ATT TCA TCA GGA AA	60	624
ZDS	F: TAA GTC HTG GCG YGT KCG YTA YAT G R: ACC TGR TTB ACT TGR TCA AGY TTG CT	55	613
CRTISO	F: AGG AGT GGA CCT TCC CAA GGA R: ATG CTC AGA AAG ATG CTT CCA T	50	238
LCYe	F: TGC AGG WTT GYB AYT GTT GCW TC R: ATR TAV GAC CAY TCC TCY TCR TA	60°	375
LCYb	F: AGT CAA AAA TGT TGC AGA AAT R: CAC CAT CCC TGC TGT TCC AC	50°	569

construct the first SNP-based genetic map of *C. pepo*.[22] This map has already been used to infer syntenic relationships between *C. pepo* and cucumber and to successfully map QTLs that control plant, flowering, and fruit traits. The third genotype 'Parador' (zucchini), which presents a yellow−orange color from the early stages of fruit development, was included to study the molecular basis of an early fruit coloration. Using these varieties, we have studied the changes in the gene expression profiles in different tissues and across different fruit development stages (differentiating skin and flesh). In addition, we have analyzed carotenoid content in the same samples to determine the correlations between transcript levels and the accumulation of specific carotenoids.

MATERIALS AND METHODS

Plant Materials. Three cultivars of *Cucurbita pepo* L. were selected on the basis of their contrasting fruit peel color: UPV196 white fruit, which corresponds to *C. pepo* ssp *ovifera* 'Scallop' (COMAV), 'MU_CU16' green fruit, which corresponds to *C. pepo* ssp *pepo* (COMAV), and cv 'Parador' yellow fruit, which corresponds to *C. pepo* ssp *pepo* (Gautier). Experiments were conducted from December 2010 to July 2011 under standard greenhouse conditions in "La Mojonera", Almería (Spain). Twelve samples of each cultivar were collected: leaf, female flower at two stages of development (before anthesis and at anthesis), and fruits at five stages of development (ovary, three-, five-, seven-, and 20 days after pollination) (Figure 1). For each fruit sample (except the first stage), exocarp tissues was cut separately into thin slices with a vegetable peeler and mesocarp was cut into small cubes after removal of seeds. The plant materials were frozen in liquid nitrogen and then stored at −80 °C for RNA isolation or freeze-dried for HPLC analysis. Both carotenoids and RNA were extracted from each sample.

Total RNA and DNA Isolation. All samples were ground to a fine powder in liquid N_2 with a pestle and mortar. Total RNA was extracted using the TRIsure reagent (Bioline, London, UK) according to manufacturer's instructions. RNA concentration and purity were determined with NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) at 260 nm absorbance. Only RNA samples with 260/280 ratio between 1.9 and 2.1 and 260/230 ratio greater than 2.0 were used for cDNA synthesis. RNA integrity was assessed by microcapillary electrophoresis with an Experion RNA StdSens Chip and the Experion bioanalyzer (BIO-RAD Laboratories, USA) showing rRNA subunits of 18S/25S peaks on the virtual gel and electropherograms. All RNA samples showed RQI values higher than 9.

Genomic DNA from *C. pepo* leaves were isolated according to DNAzol method (Invitrogen, Carlsbad, USA) and assessed on standard agarose gels.

cDNA Synthesis and Genomic Contamination. cDNA was synthesized from 1 μg of total RNA for each sample using the QuantiTec reverse transcription kit (Qiagen, Hilden, Germany) with a blend of oligo-dT and random primers according to the manufacturer's instructions. In this kit, genomic DNA is efficiently removed in a single step; nevertheless, we included a negative control to test for contaminating genomic DNA. This control contained all the reaction components except the reverse transcriptase (−RT). For each of the 36 RNA samples, a quantity equivalent to the cDNA was used in −RT control and the positive control (genomic DNA), i.e., 10 ng. The absence of amplification from gDNA in cDNA samples was tested by comparison of PCR products obtained from −RT, +RT, and gDNA with *EF1A* primers. The presence of an amplification product in the "−RT" control is indicative of contaminating DNA in the sample. In 18 samples, no amplification was detected in any control (−RT) after 40 cycles. In the remaining of samples, genomic DNA contaminating was negligible. The sample with higher gDNA contamination had a Cq value 12 cycles higher than +RT test template, therefore the "−RT" control sample contained approximately 1000-fold less target sequence (assuming 100% efficiency, $1C_q \approx$ 2-fold difference in initial template amount). Because the target template in this "−RT" control would be exclusively genomic DNA, we can conclude that 0.02% (1:4096) of the amplification in the RT sample is attributable to the genomic DNA template.

cDNA Cloning of Carotenoid Genes. *Cucurbita pepo* nucleotide sequences for *DXS*, *HDR*, *PSY*, *PDS*, *ZDS*, *CRTISO*, *LCYb*, *LCYe*, and *CHYb* were not available from the public genomic database, so different strategies were applied in order to obtain these sequences. *ZDS* was cloned using degenerate primers previously described.[26] *PSY*, *PDS*, *CRTISO*, *LCYb*, and *LCYe* were cloned using degenerate primers designed based on the conserved regions of the corresponding genes from other higher plants or specific primers designed based on conserved regions of the corresponding ESTs from Cucurbitaceae. These primer sets were designed prior to the availability of a collection of 49610 *Cucurbita* unigenes from the two *C. pepo* subspecies: subsp. *pepo* cv. Zucchini and subsp. *ovifera* cv Scallop.[30] After this publication, *DXR*, *HDR*, and *CHYb* were cloned using specific PCR primers designed from the sequence information generated in that study. A list of degenerate primers used is presented in Table 1.

For cloning, PCR amplification of gene fragments was performed using fruit cDNA as template. The following PCR conditions were used: an initial denaturation at 94 °C for 35 s, 40 cycles of denaturation at 94 °C for 35 s, annealing at 50−60 °C (depending on the primers used) for 35 s, and extension at 72 °C for 1 min, with a

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Table 2. Primers Sequences Used for qPCR and Amplicon Characteristics

gene	accession no.	forward and reverse primer sequence [5′−3′]	amplicon size (bp)	amplicon T_m (°C)	PCR efficiency (%)	regresion coefficient (R^2)
PP2A	HM594171	F: TGGTAGCATCCTTTCCCAATACA R: CATGCCCGTTCAGCTTTAGC	67	78.89	96	0.999
EF-1A	HO702383	F: GCTTGGGTGCTCGACAAACT R: TCCACAGAGCAATGTCAATGG	67	79.03	97	0.996
DXR	PU040685	F: CTTGCCCATTCTATACACCATAACA R: AAGGCGAGGCCAAGTTATTTC	70	75.41	100	0.997
HDR	PU039913	F: CGCTTAGAGGATATGGAAGTCCAA R: ACCCTTGCCAACCACTTCAA	75	76.01	100	0.998
PSY	PU055651	F: TCTCAGAGATGTTGGAGAAGATGCT R: TCTGAAATTCCTGCCTGTGCTA	81	75.96	100	0.998
PDS	PU126694	F: TAACCCCGATGAACTTTCTATGC R: CCATCTTTGAGCCATGCTTCT	80	75.65	100	0.998
ZDS	PU046841	F: CCGAGTGGTTCATGTCTAAAGGT R: GCAACTGGACCCCACATTCT	63	78.08	99	0.998
CRTISO	PU100625	F: GGTTTTACCGCCTGACACAGA R: GGGCTCCTCTAACCTTCTCCAA	70	76.11	100	0.998
LCYe	JX912283	F: TCTTGGTTTGAGTAGTCCCTGTAGTC R: GGTTGAGGTGGAAAACAATCCTT	75	74.48	100	0.999
LCYb	PU043015	F: CGGTCGAGCTACCAAAGAAGTT R: CTCTATGCGATGCCCTTTTCA	66	76.12	100	0.998
CHYb	PU058232	F: CACGACGGTCTCGTTCACAA R: TGAGCAGCAGCGACCTTTCT	80	80.51	98	0.998

final extension of 7 min at 72 °C. Taq DNA polymerase (Bioline, London, U.K.) was used in the reactions. PCR products for *PSY*, *PDS*, *ZDS*, *CRTISO*, *LCYb*, and *LCYe* were separated by electrophoresis, purified using Favorgen GEL/PCR purification kit (Favorgen Biotech Corp., Kaohsiung, Taiwan), cloned into the pGEM-T vector (Promega, Madison, USA), and sequenced. The sequence of the amplification product for each primer pair was compared in GenBank (NCBI) using the BLASTX algorithm and searches for homologous genes in other organisms. All sequences showed 87−100% identity and *E* value cutoff $<3 \times 10^{-40}$. After publication of the *Cucurbita* unigenes, the same sequences were compared in Cucurbit Genomic Database (CuGenDb, htt://www.icugi.org/) and accession numbers are indicated in Table 2. *LCYe* sequence did not correspond to any annotated sequence in CuGenDb but matched with *LCYe* showing homology with this sequence, so it was annoted and deposited in GeneBank (accession number JX912283).

Primer Design and qPCR Analysis. Specific primer pairs for qPCR amplification were designed to amplify products of 60−100 bp, with an optimal primer melting temperature (T_m) of 60 °C and GC contents between 40 and 60%. MFOLD software v3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/dnaform1.cgi) was subsequently used to evaluate the possible formation of secondary structures at the sites of primer binding, using the default settings with 50 mM Na^+, 3 mM Mg^{2+}, and an annealing temperature of 60 °C.[31]

The transcript levels of *DXS*, *HDR*, *PSY*, *PDS*, *ZDS*, *CRTISO*, *LCYb*, *LCYe*, *CHYb*, *PP2A*, and *EF1A* were analyzed using qPCR. Reactions were performed in triplicate in a 96-well plate with an Mx3000P Real-Time PCR system (Stratagene, La Jolla, USA), using SYBR Green detection chemistry. Reactions were carried out in a total volume of 15 μL that contained 7.5 μL 2× iTaq Fast SYBR Green supermix with ROX (Bio-Rad, CA, USA), 3 μL of cDNA, and 0.9 μL (300 nM) of each gene-specific primer. The following standard thermal profile was used for all PCR reactions: polymerase activation (95 °C for 3 min), amplification, and quantification cycles repeated 40 times (95 °C for 3 s, 60 °C for 30 s). Finally, a dissociation analysis of the PCR products was performed by running a gradient from 60 to 95 °C to confirm the presence of a single PCR product and specificity of the qPCR (Supporting Information Figure S1). Table 2 shows the primer sequences, amplicon sizes, melting temperatures, and PCR efficiencies.

Carotenoid Determination. All solvents and chemicals were obtained from commercial sources (Sigma and Merck). The carotenoid standards were obtained from Sigma-Aldrich and Extrasynthese.

The total amount of carotenoids was determined using a spectrophotometer (Thermo Fisher Scientific, model Evolution 300). Eight mL of ethanol were added to 200 mg of freeze-dried sample and homogenized in a test tube followed by a 2000*g* centrifugation for 5 min. This procedure was repeated until the sample became colorless. An aliquot was taken from the supernatant for measurement of optical density at 663, 645, and 470 nm in the spectrophotometer. The total carotenoid content was calculated according to the equations of Lichtenthaler and Wellbur.[32]

To determine the amount of β-carotene and lutein, a 200 mL finely ground sample was extracted using a slight modification of the method described by Tadmor.[33] The carotenoids were extracted from the rehydrated sample with 8 mL of ethanol containing 1 mg mL^{-1} butylated hydroxytoluene (BHT) using a Polytron homogenizer. All steps were carried out in darkness or under gold fluorescent light to

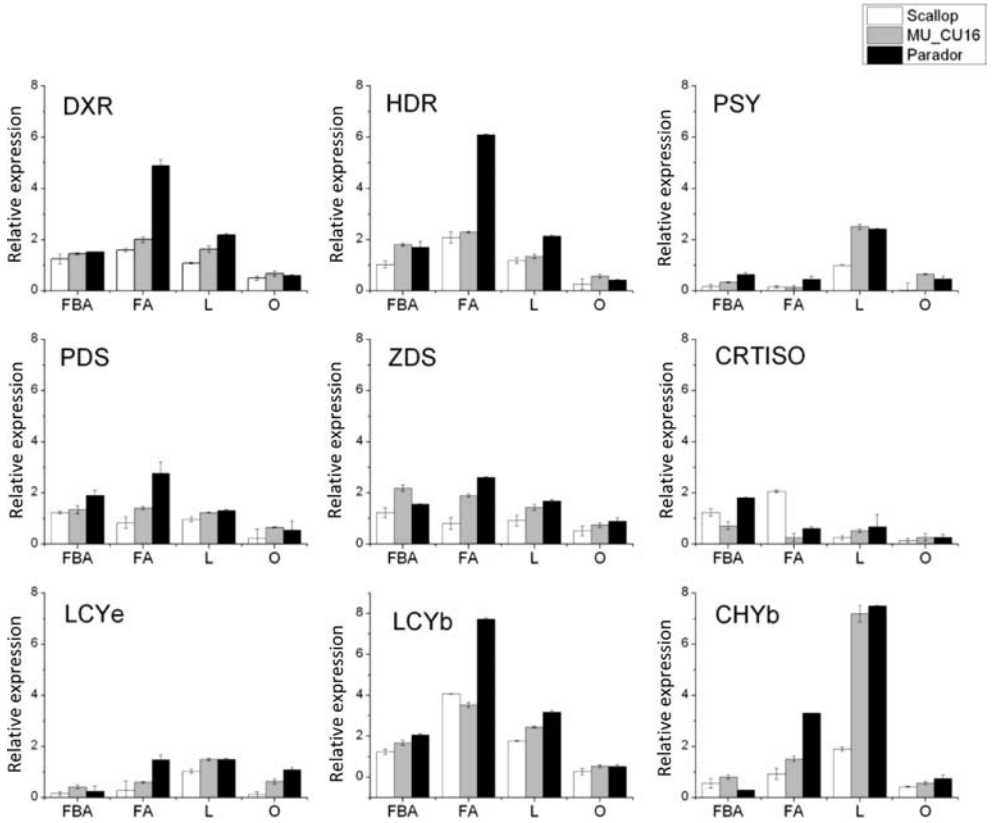

Figure 2. Relative expression of nine carotenoid pathway genes in different organs of three varieties of *Cucubita pepo* L. as determined by qPCR: flower before anthesis (FBA), flower at anthesis (FA), leaf (L), and ovary (O). Error bars represent the mean from three technical replicates. The expression levels of *PP2A* and *EF1A* were used to normalize the mRNA levels for each sample.

prevent possible photodegradation of products. To hydrolyze esterified carotenoids that might complicate the chromatographic determinations, we saponified the samples.[34] One mL of 40% w/v KOH methanolic solution was added to each tube, and the samples were saponified for 10 min at 85 °C. The samples were cooled in an ice bath, and 2 mL of ice-cold water was added. The suspensions were extracted twice with 2 mL of hexane by vigorous vortexing followed by a 2000*g* centrifugation for 10 min at room temperature. The upper hexane layers were pooled and evaporated to dryness in a Savant SpeedVac apparatus. Immediately before injection, the carotenoids were dissolved in 400 μL of an acetonitrile/methanol/dichloromethane (45:20:35 v/v/v) solution, filtered through a 0.22 μm PTFE syringe filter (Millipore) directly into an amber flask, and 10 μL were injected into the chromatograph. The analyses were carried out on a HPLC apparatus equipped with binary pump, in-line vacuum degasser, autosampler injector, a Waters Symmetry C18 column (4.6 mm × 150 mm, 5 μm), and a dual λ absorbance detector (model 2487), controlled by a Breeze workstation. The initial mobile phase consisted of acetonitrile/methanol (97:3, v/v/v) containing 0.05% (v/v) triethylamine. We used a linear gradient of dichloromethane from 0 to 10% in 20 min at the expense of acetonitrile, and then the dichloromethane was kept constant at 10% until the completion of the runs. The flow rate was 1.0 mL/min, while the column temperature was 30 °C. Colored carotenoids were detected at 450 nm.

Compounds were identified by comparison of retention times, coinjection with known standards, and comparison of their UV−visible spectra with authentic standards (β-carotene and lutein). Quantification was carried out by external standardization. Full standard curves were constructed with five different concentrations for each carotenoid in triplicate. The curves passed through or very near the origin, were linear, and bracketed the concentrations expected in the samples. Calculations of fresh weight, dry weight, and moisture content were based on weights determined before and after freeze-drying samples at −55 °C for 7 h. days.

Statistical Analysis. Expression levels of the target genes were calculated using the advanced relative quantification model with efficiency correction, multiple reference gene normalization, and use of error propagation rules.[35] On the basis of previous results, two genes (*EF1A* and *PP2A*) were selected as reference genes to normalize.[36] Standard error was calculated using three technical repetitions.

Transcript levels, as measured by qPCR, were correlated with total carotenoid content in exocarp and mesocarp of fruit development in colored varieties. Pearson correlation (r) analysis was performed and tested for statistical significance using Statistix 8.0 (Analytical Software, Tallahassee, USA).

RESULTS

Analysis of Transcripts in Different Organs. Transcript levels of *DXS*, *HDR*, *PSY*, *PDS*, *ZDS*, *CRTISO*, *LCYb*, *LCYe*, and *CHYb* genes, measured by qPCR in the 36 samples, were detected in all samples including those from the white fruit cultivar. Overall, transcript levels in fruit were highest in skin compared with flesh and expression increased during fruit development for most of the genes. Expression of the nine genes was examined in different organs for the three cultivars ('Scallop', 'MU_CU16', and 'Parador'). The analysis in flower before anthesis (FBA), flower at anthesis (FA), leaf (L), and ovary (O) showed different expression patterns depending on the gene. The results are presented in Figure 2.

The lowest expression levels for all genes were found in the ovary, with the variety 'Scallop' having the minimum level. Expression of *CRTISO* was up-regulated in flowers of 'Scallop', however, as compared to the levels in the other varieties. *DXR*, *HDR*, *PDS*, and *LCYb* transcripts exhibited similar expression patterns; the highest expression levels were obtained for flower

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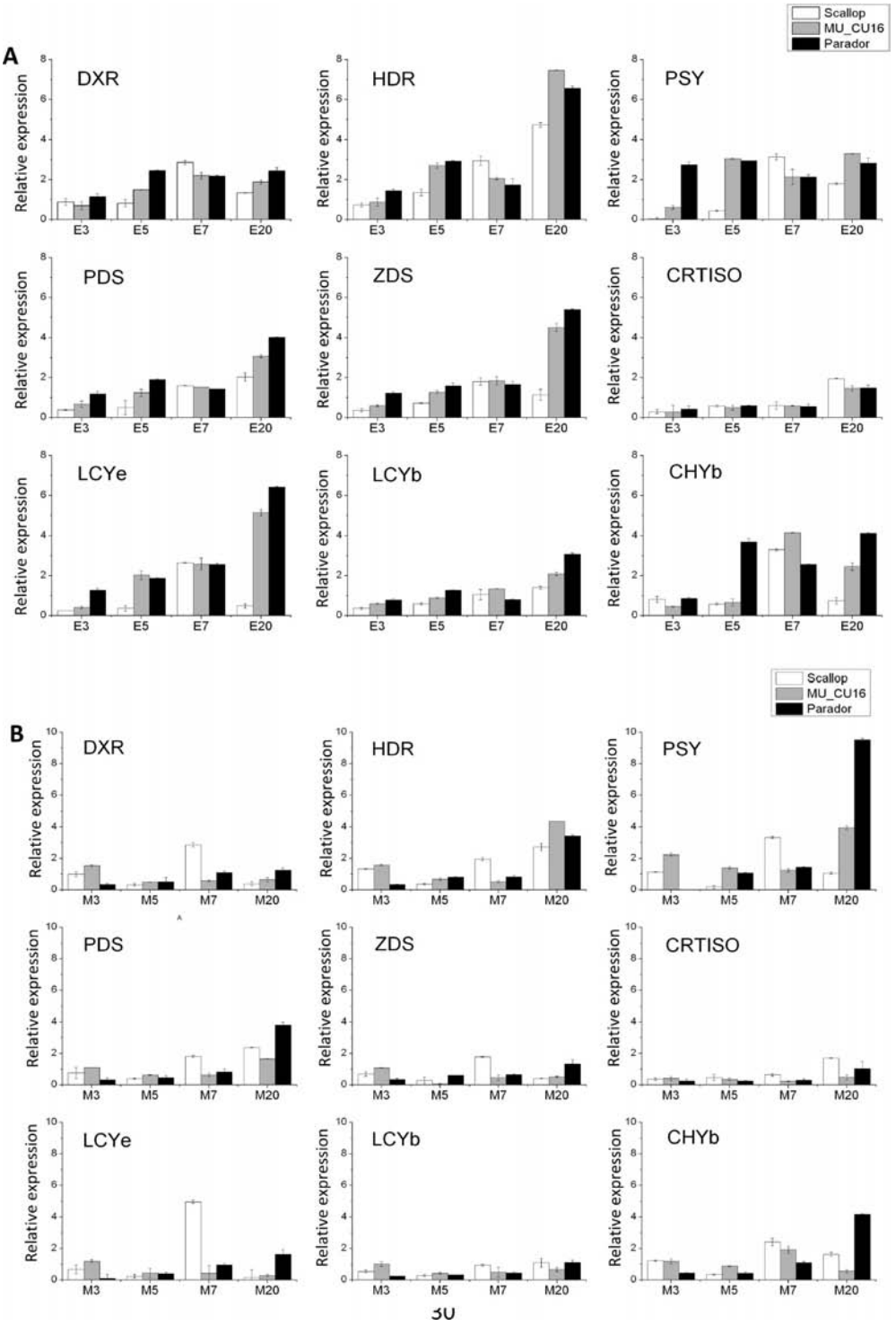

Figure 3. Relative expression of nine carotenoid pathway genes in exocarp (A) and mesocarp (B) of fruit from three cultivars of *Cucurbita pepo* L. during development. E3, E5, E7, and E20 and M3, M5, M7, and M20 represent different stages (3, 4, 5, and 20 days after pollination). Error bars represent the mean from three technical replicates. The expression levels of *PP2A* and *EF1A* were used to normalize the mRNA levels for each sample.

at anthesis (FA), principally for the yellow−orange fruit cultivar 'Parador' followed by the green fruit cultivar 'MU_CU16'. For *PSY*, *LCYe*, and *CHYb*, the highest transcript levels were detected in leaves. In the case of *CHYb*, message accumulation was about 25-fold higher in the leaf of 'Parador' than in the preanthesis flower in the same cultivar. *CHYb* showed a prominent difference of expression values in the leaves of colored fruit varieties compared with those from the white fruit variety.

Analysis of Transcripts during Fruit Development. Expression levels of all genes were higher in exocarp compared with mesocarp, with the exception of *CRTISO*, which showed a similar pattern in both the skin as the flesh (Figure 3). Most of the genes showed increasing transcript levels during fruit

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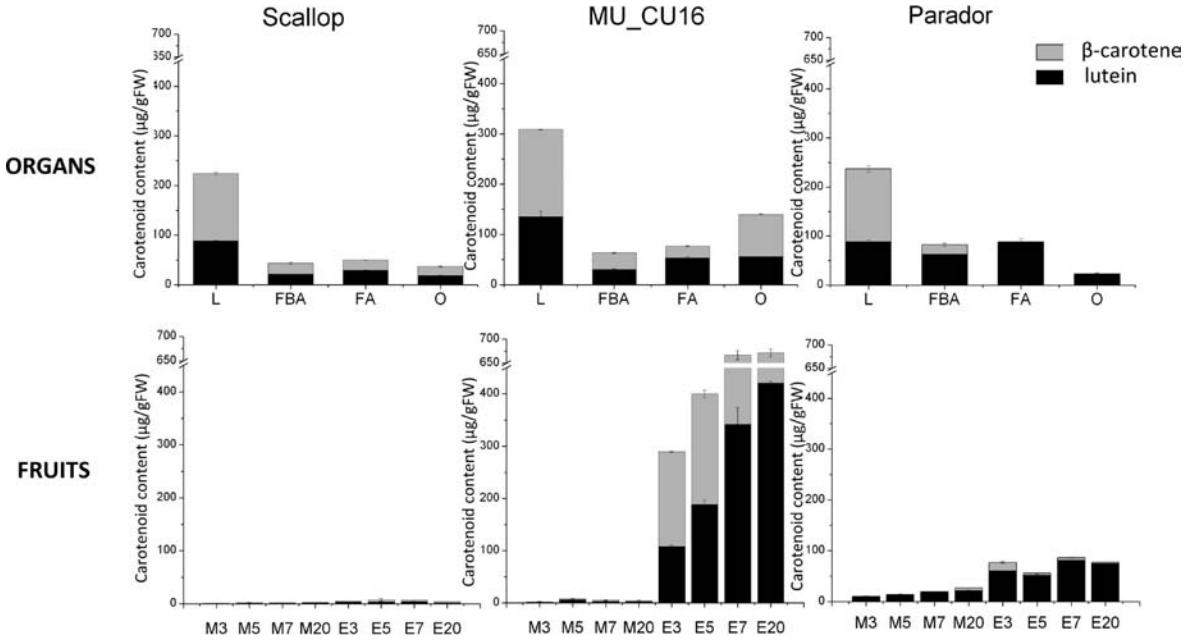

Figure 4. Carotenoid content in three varieties of *Cucurbita pepo* L.: 'Scallop' (white), 'MU_CU16' (green), 'Parador' (yellow–orange). Error bars represent ± SD of the three replications.

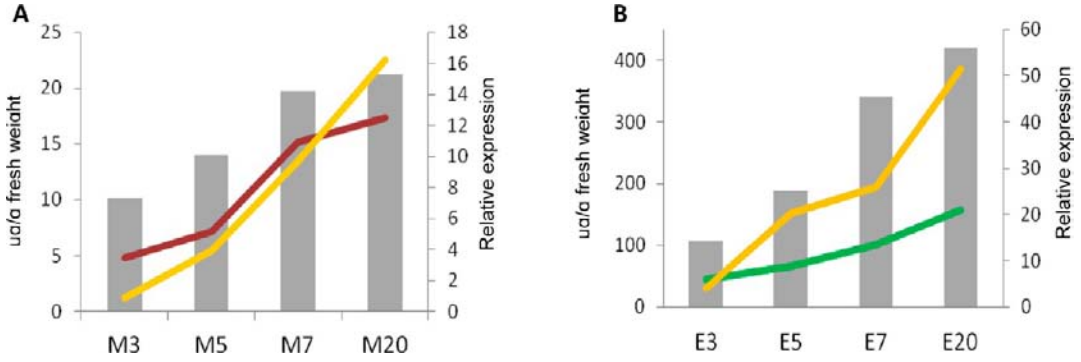

Figure 5. Correlation between lutein content (gray bars) and expression levels of *DXR* (red line), *LCYb* (green line) and *LCYe* (orange line) during fruit development. Figure (A) shows the correlation in fruit flesh of yellow–orange variety 'Parador'. Figure (B) shows the correlation in fruit skin of green variety 'MU_CU16'.

development, with this pattern being more evident in skin than in flesh. In ripe fruit exocarp (E20) (Figure 3A), *HDR*, *PDS*, *CRTISO*, and *LCYb* expression were at their highest for all three cultivars, while *ZDS* and *LCYe* expression in ripe fruit were the highest in 'Parador' and 'MU_CU16' but not in 'Scallop' (white cultivar). At the earliest stage of development (E3), transcripts levels for all genes except *PSY* were lowest. For *PSY*, expression in the exocarp of 'Parador' was higher compared with 'MU_CU16' and 'Scallop', with a 55-fold of difference between yellow–orange and white fruit variety.

In 'Parador', the fruit mesocarp (Figure 3B), transcript levels of all genes increased gradually during fruit development. *PSY* showed the greatest change in expression in that at the beginning of fruit development (3 days after pollination) transcripts were practically negligible, however by E20 stage (20 days after pollination), the difference was about 9-fold compared with fruit at E5 stage (5 days after pollination).

Carotenoid Accumulation and Relationship with Gene Expression. The color changes during fruit development in *C. pepo* were different according to the variety. In 'Parador', the fruit flesh was yellow–orange in the early development stages and then turned orange by M20 stage (20 days after pollination). In 'MU_CU16', this change was opposite, fruit flesh in early development stages was a greenish–yellow color, which was more intense than at M20 stage where the flesh was a lighter color. Changes in flesh color of the white variety were less obvious during development; ovary color was green and the flesh became whiter as the fruit was developed.

The total amount of carotenoids and chlorophyll were determined using a spectrophotometer. As it is known that the principal carotenoids found in *C. pepo* are lutein and *β*-carotene,[28,37] we examined levels of these compounds in mesocarp and exocarp of all stages of fruit development and also in different organs for the three varieties by HPLC (Figure 4). The organs showed less difference in carotenoid content among three varieties than fruits. The principal carotenoids in flowers of all three varieties were lutein followed by *β*-carotene. At anthesis (FA), the ratio of lutein to *β*-carotene increased. In leaves (L), the carotenoid content was highest for 'MU_CU16' followed by 'Parador' and 'Scallop' (308.6 > 237.9 > 224.3 μg/g fresh weight). When comparing ovary of the three varieties,

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'MU_CU16' showed the highest values (140 μg/g fresh weight).

In fruits, the carotenoid level in skin of the green variety 'MU_CU16' was higher than in the yellow−orange variety 'Parador'. In general, carotenoid accumulation during fruit development increased. In contrast, the flesh of the yellow−orange variety accumulated more carotenoid than the green variety, showing also an increase in concentration with development. Small amounts of carotenoids were detected in both fruit skin and flesh of the white cultivar 'Scallop'. Supporting Information Figure S2 shows typical HPLC chromatograms of the three varieties.

The relationships between carotenoid content and gene expression levels were studied, and Pearson correlation was performed between relative gene expression for all genes during fruit development and total carotenoid concentration. The same analysis was repeated with each carotenoid, lutein, or β-carotene. Correlation analysis showed highly significant correlation for *DXR* and *LCYe* expression in 'Parador' fruit mesocarp with lutein accumulation ($r = 0.98$, $P < 0.02$; $r = 0.96$, $P < 0.04$, respectively). In comparison, in 'MU_CU16' exocarp, higher correlation was found between expression of *LCYb* and *LCYe* and lutein accumulation ($r = 0.96$, $P < 0.03$; $r = 0.93$, $P < 0.06$, respectively) (Figure 5). In the white fruit variety 'Scallop' and the flesh of green fruit 'MU_CU16', where total carotenoid content were low, no significant correlations were observed.

DISCUSSION

The intensive colors of some flowers, fruits, and leaves are due to a combination of various pigments including carotenoids.[4] *C. pepo* shows a significant variation in color between different varieties, suggesting that mechanisms regulating the accumulation of carotenoids vary among varieties. With the aim of understanding which mechanisms are regulating carotenogenesis in *C. pepo*, we have analyzed carotenoid expression of two isoprenoid and seven carotenoid genes in different organs of *C. pepo* and fruit developmental stages. The levels of the principal carotenoids, and correlation with gene expression patterns were also analyzed.

The majority of the carotenoids in all organs were lutein and β-carotene. The high levels of these two carotenoids are similar to those described in other fruit crops and flowers such as mango, papaya, kiwifruit, or japanese morning glory.[38−40] While the white color of the variety 'Scallop' is due to the absence of carotenoids, yellow−orange color of skin in 'Parador' is mainly attributed to lutein. In the green fruit variety, a high chlorophyll content would mask the elevated content of lutein and β-carotene. Larger quantitative differences were detected among the fruits of the three varieties than in the leaves. Other than leaves, where carotenoids are essential for photosynthesis, the highest levels of these compounds were found in the skin of the green variety 'MU_CU16'. In preanthesis flowers of all three varieties, the principal carotenoid was lutein following of β-carotene. When the flowers open, lutein increases, while β-carotene content decreases. In contrast to leaves and flowers, the varieties differ dramatically in the accumulation of carotenoids in their fruits, especially in the skin. Only the skin of the green variety showed higher levels of carotenoid than flowers and leaves.

In our study, the highest β-carotene content in flesh of the three cultivars (2 μg/g) was lower than those reported for *C. pepo* cultivars by Azevedo-Meleiro and Rodriguez-Amaya[28] and Murkovic et al.[37] (5.4 and 23 μg/g, respectively). However, the highest lutein content in flesh (20 μg/g) we found was slightly higher than results obtained by these authors (10 and 18 μg/g). Results by Nakkanong et al.[29] showed higher levels of both compounds in pumpkin, with the interspecific inbred lines showing the highest lutein and β-carotene content (about 56.6 and 30.5 μg/g, respectively). The high levels in pumpkins may be the result of a long harvest period during which the ripening process continues.

The higher level of carotenoids in the exocarp compared to the mesocarp of the three varieties is correlated with the expression levels of the analyzed genes. Flesh of fruit species usually show lower total carotenoid content than the skin, and gene expression levels are concomitantly decreased in the flesh.[26,41] In this study, for the majority of the genes analyzed, expression levels in leaves and flowers were also consistent with high carotenoids content in these organs. Striking results were observed for transcripts levels of *PSY*. Although high levels of carotenoids were found in flowers, very low *PSY* transcripts levels were found in this organ compared with other genes in the pathway. Much higher expression levels for *PSY* were found in ripe flesh of the orange variety than in any of the other varieties. Taking into account that *PSY* is generally accepted as the most important regulatory enzyme in the pathway and that expression can be tissue-specific,[15] these results point to the possibility of having another *PSY* isoform in summer squash with expression patterns differing according to tissue. In tomato, high levels of expression of *PSY1* were found in red and pink fruit, whereas higher levels of *PSY2* were found in petals and green tissues such as leaves and sepals.[42] In other Cucurbitaceae such as *Cucumis melo*, *PSY1* and *PSY2* exhibit distinctive expression patterns in different tissues as well as during melon fruit development, with *PSY2* transcript accumulation highest in root.[43] In other squashes, the expression of *PSY1* was correlated with the amount of carotenoid present, suggesting that *PSY1* activity is related with final carotenoid concentration in ripe fruit.[29]

With respect to carotenoid accumulation in the skin and flesh of *C. pepo* fruit, it should be noted that transcripts of all genes analyzed were detected across all samples, including tissues of the white variety where lutein and β-carotene were almost undetectable. In skin, *LCYe*, *ZDS*, and *CHYb* showed the highest difference in relative expression when comparing the colored and the white varieties at 20 days after pollination (E20), coinciding with a higher content of lutein. In flesh, although the color variation among the three cultivars is not as evident as in the skin, and differences in the levels of transcripts are not so marked, significant correlation values have been found for carotenoid content and expression levels along developmental stages for the *LCYe* and *DXR* in the yellow−orange variety 'Parador', indicating a possible implication of these genes in the color development. In skin of the green fruit variety 'MU_CU16', high correlation was found for transcripts of *LCYb* and *LCYe* with lutein levels. These results suggest that different regulatory mechanisms may be controlling carotenoid accumulation in skin and flesh. Moreover, it could also be deduced that *LCYe* can be mainly responsible for the regulation of carotenoid accumulation in fruit of *C. pepo* because its expression appears to be related to the trait in both tissues. Considering the accumulation of lutein detected in *C. pepo* fruit, it should be mentioned that similar results have been found in organs of other plants that also accumulate lutein. In this sense, high expression of the gene encoding *LCYe* has been found in the yellow cultivar of carrot, consistent with the

accumulation of lutein.[44] In citrus fruits, lutein accumulation and high expression of *LCYe* during the green stage in the flavedo has been reported.[26,45] In apple, the expression of *LCYe* was also highly correlated with carotenoid content in the skin of the fruit and was down-regulated during fruit development when lutein content was reduced.[46] In *C. pepo*, both in the skin of the green fruit variety and the flesh of the yellow−orange variety, accumulation of lutein increases during fruit development while *LCYe* is up-regulated. Transformation experiments support the hypothesis that lutein accumulation is largely rate-determined by *LCYe* expression.[47,48] Apart from the *LCYe* gene, *DXR* and *LCYb* gene expression showed a significant positive correlation with lutein content and subsequently may also be involved in the regulation process.

As in the study of carrot,[44] the differential transcriptional regulation of carotenogenic genes is not the principal cause of the absence of pigmentation of the white variety. Apart from regulation at the level of transcription, other mechanisms could explain the absence of the measured carotenoids. The fact that transcripts of two representative genes of the MEP pathway *HDR* and *DXR* were detected suggests the availability of substrates; therefore, the absence of precursors could not explain the lack of carotenoid in white variety. Carotenoid degradation could explain the differential accumulation. In white chrysanthemum and peach, the differential expression of carotenoid cleavage dioxygenases (CCDs) are likely to be the major determinant in the accumulation of carotenoids.[49,50] Accumulation of carotenoid also can be affected by sink capacity. Chromoplasts are the major storage structures of carotenoid; in the white-fleshed loquat, the failure to develop normal chromoplasts seems to be the most convincing explanation for the lack of carotenoid accumulation.[41] In other plant species, genetic variation in carotenoid biosynthesis enzymes may explain the differences in carotenoid compounds.[51,52] Therefore, future studies could elucidate the differential content of carotenoids in fruit of *C. pepo*.

This work can be considered as a first step in understanding the mechanisms of regulation of carotenoids in summer squash. Further work will be needed to examine genes of interest and the possibility of allelic differences, studies of rate of degradation by carotenoid cleavage dioxygenases or post-transcriptional regulation, and its relationships to carotenoid accumulation. Taking into account that two of these genotypes, 'MU_CU16' and 'Scallop' are the parental lines of the segregating population used to construct the first SNP-based genetic map of *C. pepo*;[22] in the future, polymorphic markers for these genes could be developed for genetic location in the map and subsequent identification of potential QTLs associated with carotenoid content in the species.

From the nutritional point of view, three major sources of carotenoid can be found in *C. pepo*. The fleshy fruit pericarp normally comprises the major nutritive tissue. In this sense, the edible skin of 'Parador' and further 'MU_CU16' might provide high concentrations of lutein and β-carotene to the diet. Although it is generally thought that flesh of summer squashes have low carotenoid content, in the yellow−orange flesh of 'Parador' fruit, lutein content is high compared with the others varieties. Considering these findings together with the fact that the skin of summer squash fruit is often removed when processed, the flesh of yellow-orange variety 'Parador' can be considered as an important product to be commercially enhanced in markets. In the same way, edible flowers of *C. pepo* that are being used in the culinary arts for flavor and garnish can contribute to the nutritional value in foods.

■ ASSOCIATED CONTENT

Supporting Information

Dissociation curves of two isoprenoid and seven carotenoid pathway genes, showing single peaks. Example of three typical HPLC chromatograms for the three varieties: Scallop, MU_CU16, and Parador (E20). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

HDR, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; CHYb, β-ring hydroxylase; CRTISO, carotene isomerase; LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; PDS, phytoene desaturase; PSY, phytoene synthase; HPLC, high-performance liquid chromatography; qPCR, quantitative real-time PCR; Cq, quantification cycle

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