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Carotenoid Accumulation and Carotenogenic Gene Expression during Fruit Development in Novel Interspecific Inbred Squash Lines and Their Parents

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Supporting Information

ABSTRACT: Carotenoid levels and composition during squash fruit development were compared in *Cucurbita moschata*, *Cucurbita maxima*, and two lines of their interspecific inbred lines, namely, Maxchata1 and Maxchata2. Eight genes associated with carotenoid biosynthesis were analyzed by quantitative RT-PCR. The two squash species and their interspecific inbred lines exhibited different qualitative and quantitative carotenoid profiles and regulatory mechanisms. *C. moschata* had the lowest total carotenoid content and mainly accumulated α-carotene and β-carotene, as expected in a fruit with pale-orange flesh. Low carotenoid content in this species was probably due to the comparatively low expression of all genes investigated, especially *PSY1* gene, compared to the other squashes. The predominant carotenoids in *C. maxima* were violaxanthin and lutein, which produced a corresponding yellow flesh color in mature fruit. The relationship between the expression of the *CHYB* and *ZEP* genes may result in almost equal concentrations of violaxanthin and lutein in *C. maxima* at fruit ripening. In contrast, their interspecific inbred lines principally accumulated lutein and β-carotene, leading to orange flesh color. The *PSY1* gene exhibited higher expression levels at earlier stages of fruit development in the Maxchata lines, potentially triggering the increased carotenoid accumulation seen in these fruits. Likewise, the higher transcription level of *CHYB* gene observed in the two interspecific inbred lines might be correlated with high lutein in these hybrids. However, this study could not explain the observed β-carotene accumulation on the basis of gene expression.

KEYWORDS: *Cucurbita, interspecific inbred line, carotenoids, gene expression, fruit development*

INTRODUCTION

Carotenoids are plant pigments responsible for a wide range of yellow, orange, and red tissue coloration.[1] In higher plants, carotenoids support light harvesting and protect against oxygen species; hence, they are important in the maintenance of photosynthetic tissues. In nonphotosynthetic tissues, carotenoids can contribute to the color of flowers, fruits, and other storage organs such as roots and tubers.[2] They also serve as precursors for the production of the plant hormone abscisic acid (ABA)[3] as well as flavor and aromatic compounds in flowers and fruits.[4] There is considerable interest in dietary carotenoids due to their importance in human nutrition and health. Carotenoids are vitamin A precursors in human and animal diets.[5] Vitamin A deficiency (VAD) can lead to an irreversible form of blindness called xerophthalmia.[6,7] The antioxidant properties of carotenoids can prevent or reduce the incidence of certain diseases such as cancer, cardiovascular disease, and ocular diseases.[8] Moreover, carotenoids may offer protection against UV irradiation.[9] Commercially, carotenoids are also used as food colorants and nutritional supplements and in cosmetics and pharmaceuticals.

Carotenoid biosynthesis in plants is well described. Two molecules of geranylgeranyl pyrophosphate (*GGPP*) (C40) are converted to phytoene (C20) by phytoene synthase (*PSY*).[10] Colorless phytoene is then catalyzed into lycopene by two desaturase enzymes, phytoene desaturase (*PDS*) and ζ-carotene desaturase (*ZDS*), via ζ-carotene.[11] The subsequent cyclization of the linear lycopene represents a branching point, one with an ε-ring and one with a β-ring. The first branch is catalyzed by lycopene β-cyclase (*LCYB*) and lycopene ε-cyclase (*LCYE*) to α-carotene. Thereafter, α-carotene is hydroxylated into lutein by β-ring hydroxylase (*CHYB*) and α-ring hydroxylase (*CHXE*). In the second branch, β-carotene is produced from lycopene by *LCYB*.[6] β-Carotene is then hydroxylated to form zeaxanthin by *CHYB* and then is catalyzed to violaxanthin and neoxanthin by zeaxanthin epoxidase (*ZEP*). These two compounds are catalyzed into apocarotenoids by carotenoid cleavage dioxygenases (*CCD*) (see Figure 1).[4]

The regulation of carotenoid biosynthesis has been widely investigated in several organs of many plant species. Tomato has been the model system for many studies of carotenogenesis in ripening fruits. The accumulation of lycopene results from increases in *PSY* and *PDS* expressions[12,13] and decreases in *LCYB* and *LCYE* transcripts.[13,14] In the juice sac of citrus fruits, color change from green to orange was caused by the transition

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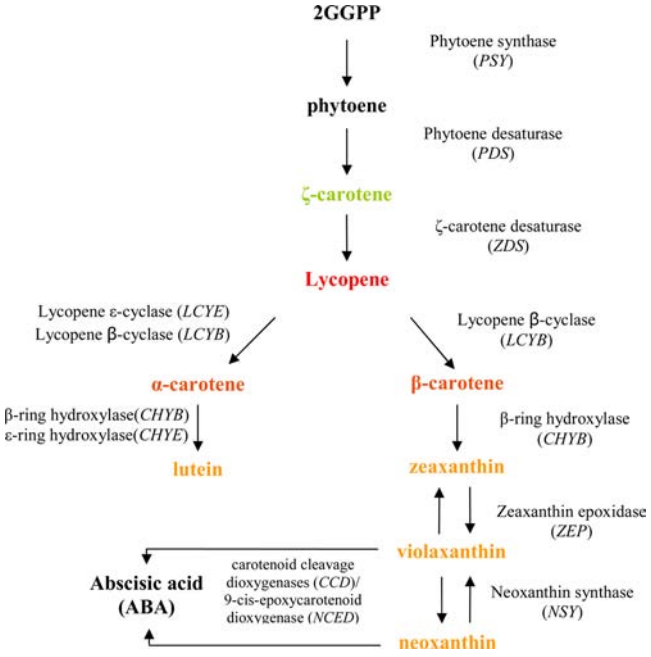

Figure 1. Simplified schematic of plant carotenoid biosynthesis. Major products in the carotenoid pathway are represented in bold, and enzymatic conversions are shown by arrows.

from *β,ε*-carotenoids to *β,β*-carotenoid biosynthesis resulting from the absence of transcripts for *LCYE* concomitant with increases in *PSY*, *PDS*, *ZDS*, *LCYB*, *CHYB*, and *ZEP* transcripts.[15] In papaya, the *LCYB* gene was cloned, and its expression in yellow- and red-fleshed papaya was observed. This gene was down-regulated during the fruit ripening process.[16] *β*-Carotene was the principal carotenoid found in kiwifruit, and its accumulation was controlled by the increased expression of *LCYB* and *ZDS* genes along with the down-regulation of *LCYE* and *CHYB* genes.[17]

The family Cucurbitaceae includes many edible crops such as cucumber, melon, watermelon, and squashes. The genetic regulation of carotenoid biosynthesis in watermelon has been extensively studied in relation to the development of different colors of the fruit flesh.[20] A functional marker was developed to discriminate between watermelons with yellow and red flesh, on the basis of the SNPs found in the coding region of the *LCYB* gene.[21] The genes expressed during carotenoid biosynthesis, and the carotenoid compositions in fruits with differing flesh color were also determined. In red and pink watermelons, the reduction of *LCYB* and *CHYB* transcription levels may be consistent with massive accumulations of lycopene and *β*-carotene during fruit ripening. Moreover, watermelons with yellow flesh showed high expression levels of *CCD* gene that might lead to lower carotenoid contents.[11] *Cucurbita* species vary widely in the color of their fruit flesh, ranging from white to yellow and deep orange.[22] The flesh color is one of the most important quality traits for consumer preference or acceptance, as well as nutritional value. Intense yellow or orange flesh is an especially attractive quality in pumpkin and winter squash.[23] The intensity of flesh color is positively correlated with total carotenoid content in different cultivars.[24,25] Pumpkin and squash are important vegetable sources of carotenoids. Hence, it is important to understand the mechanisms regulating carotenoid biosynthesis to help in the development of breeding strategies for increasing the important nutritional and economic value of these plants. The carotenoid composition of mature fruit has been studied in many different *Cucurbita* species.[26−28] However, the carotenoid composition of squashes during fruit development and the molecular basis of carotenoid accumulation have not been studied. The objective of this study was to investigate the transcriptional regulation of carotenogenic genes responsible for different carotenoid compositions in squash fruits. Hence, the pattern of carotenoid accumulation and the expression of genes related to carotenoid biosynthesis were compared during fruit development of *Cucurbita moschata*, *Cucurbita maxima*, and their interspecific inbred lines.

MATERIALS AND METHODS

Plant Materials. The squash species used in this study were *C. moschata*, *C. maxima*, and two of their interspecific inbred lines. The interspecific inbred line was initially developed using *C. moschata* and *C. maxima* as male and female parent, respectively. The F_1 was twice backcrossed with *C. maxima*, ollowed by five generations of self-crossing. The four types of squash were grown in a greenhouse at Huajiachi campus, Zhejiang University, China. Hand pollination was performed at flowering time. Male and female flowers that would bloom during the next day were folded with a plastic clip in the evening. Then, the next day between 8:00 and 10.00 a.m., the flowers were self-pollinated, closed with clips again, and tagged to identify the start of development. Fruits were collected at different developmental stages [0, 12, 24, 36, 48, and 60 days after pollination (DAP)]; the fruits were peeled and deseeded, frozen immediately in liquid nitrogen, and then stored at −80 °C until analysis. Three replicates of each sample were performed.

Carotenoid Extraction. Carotenoids were extracted according to the methods described by Fraser et al.[29] and Xu et al.[30] All samples were analyzed in triplicate. Briefly, fruit flesh portions (mesocarp) were homogenized under liquid nitrogen, and 200 mg samples of fruit flesh powder were placed into a 2 mL centrifuge tube. The samples were extracted with 1.4 mL of chloroform/methanol/50 mM Tris buffer, pH 7.5, containing 1 M NaCl (2:1:1, v/v/v) in the tube, followed by centrifugation at 4 °C for 10 min at 10000 rpm. The chloroform phase was then transferred to new tubes, and the aqueous phase was re-extracted with 700 μL of chloroform until colorless. The pooled chloroform extracts were dried under nitrogen flow. The residues were then dissolved in 50 μL of diethyl ether and 350 μL of 6% (w/v) KOH in methanol. The mixture was incubated at 60 °C for 30 min in darkness for saponification, then 350 μL of water and 700 μL of chloroform were added. After centrifugation, the chloroform phase was recovered and partitioned with water until the aqueous phase became neutral. The chloroform extracts were dried under a gentle stream of nitrogen and then stored at −20 °C until HPLC analysis.

HPLC Analysis of Carotenoids. HPLC analysis of carotenoids in fresh fruit samples of squashes was carried out using a Waters Alliance 2695 system (Waters Corp., Milford, MA, USA) consisting of a 2695 separation module and a 2996 photodiode array detector, equipped with a 250 mm × 4.6 mm i.d., 5 μm, YMC reverse-phase C_{30} column and a 20 mm × 4.6 mm i.d., YMC C_{30} guard (Waters). Before analysis, the dried residues were dissolved in 50 μL of ethyl acetate. A 10 μL aliquot of each sample was injected into the HPLC column. Chromatography was carried out at 25 °C with an elution program as previously described.[30] Carotenoids were identified on the basis of their retention times and spectroscopic characteristics compared with standards. Individual carotenoid concentrations were calculated by converting peak areas to molar concentrations by comparison with carotenoid standards of known concentration run on a HPLC. All carotenoid standards were kindly provided by the Laboratory of Fruit Molecular Physiology and Biotechnology, Huajiachi Campus, Zhejiang University. Full details of these methods for carotenoid quantification have previously been reported elsewhere.[31]

Total RNA Isolation and cDNA Synthesis. The same plant material was used for total RNA isolation as for carotenoid analysis. Total RNA was extracted from frozen fruit flesh tissue using the total

RNA isolation kit (Omega, USA) according to the manufacturer's instructions. To eliminate genomic DNA, extracted RNA was treated with the RNase-free DNase supplement provided with the kit. UV absorption spectrophotometry and gel electrophoresis were performed to test RNA quality and purity as described by Sambrook et al.[32] cDNA was synthesized from 2.0 μg of total RNA using 50 μM oligo-dT primer and reverse transcriptase M-MLV (RNase H$^-$) following the manufacturer's procedures (Takara, Japan). To confirm the absence of genomic DNA, all cDNA samples were tested with specific primers of the gene encoding 18S rRNA. The primers were designed to anneal to sequences in exons on both sides of an 18S intron to discriminate the size of amplicons generated from cDNA and genomic DNA templates.

Quantitative Real Time PCR Analysis. The transcription levels of *PSY1*, *PDS*, *ZDS*, *LCYB*, *LCYE*, *CHYB*, *ZEP*, and *CCD1* were analyzed by using quantitative real time PCR with the ABI StepOne PCR System (Applied Biosystems, Foster City, CA, USA). All reactions were performed using the Fast Start Universal SYBR Green Master Mix (Roche) following the manufacturer's recommended procedures. For primer selection, PCR was performed to test the specificity of the primers, based on the analogous *Cucurbita* coding sequences from gene cloning results, which were available in the GenBank nucleotide database (see Supporting Information , Table S1) with T_m at 60 °C. PCR products were run on 2% (w/v) agarose gels containing GoldView for visualization after electrophoresis in 0.5% TBE buffer. The chosen primers used for quantitative real time PCR are listed in Table 1. To transform fluorescent intensity measurements into relative mRNA levels, a 5-fold dilution series of a cDNA sample was used to generate a standard curve. PCR efficiencies (E) were calculated for each gene using the slopes given in Light Cycler software for the standard curve, from serial dilutions according to the equation $E = 10^{-1/\text{slope}}$ as described by Rasmussen.[33] *C. moschata* fruit at 0 DAP was chosen as a calibrator for each group of samples and assigned a nominal value of 1.0. Three replicate PCR reactions were performed using about 1 μL of cDNA (50 ng/μL of total RNA), 0.3 μM of each specific primer, and 10 μL of 1× SYBR Green PCR master mix (Roche) in a 20 μL volume. A negative control was included using water as a template in each reaction. The reaction mixtures were initially denatured at 95 °C for 10 min, followed by a quantification program of 40 cycles of 95 °C for 15 s and 60 °C for 60 s. At the end of each run, melting curve analyses were conducted following the instrument instructions by slowly increasing the temperature from 60 to 95 °C to ensure the specificity of the primer and the purity of the amplified product. Relative expression levels were calculated using the ΔΔCt method[34] and normalized Ct data obtained from a target gene with Ct values from 18S rRNA gene as an internal control. The Ct values presented are the means of three independent biological replicates, and each reaction had three technical replicates.

Table 1. Primers Used for Quantitative Real Time PCR Analysis

gene	direction	sequences (5′ → 3′)	amplicon size (bp)
PSY1	forward	CCAAACATTGGACGAGATTG	113
	reverse	GGAGAGACCTTGCATAAGCC	
PDS	forward	GTTTAGCAGGAGTCCGCTTC	119
	reverse	AAATCCACTCCTCTGCAGGT	
ZDS	forward	CAACGGATGGGTTACAGAGA	111
	reverse	GCAAAGCATGAGAAATCTGC	
LCYB	forward	CAACTGCCTGTTCTTCCTCA	112
	reverse	CAACAATAGGTGCTGCTGCT	
LCYE	forward	GATCCTTGCCAAACACAGAG	109
	reverse	TTTGGAGCCTCAGACAGAGA	
CHYB	forward	CTGGCCTTGGAATTACTGT	109
	reverse	TTCTGAAGTAGGGCACGTTG	
ZEP	forward	ATCTCCTGGTTGGAGCTGAT	117
	reverse	CTGGTATGAAGTCGGCGATA	
CCD1	forward	TATGCGATGGTTTGAGCTTC	120
	reverse	CTGACCATGTCCAAGTCTGG	
18s	forward	CCCTCCAATTGATCCTCGT	120
	reverse	CGCAAATTACCCAATCCTG	

RESULTS AND DISCUSSION

Color Changes of Squash Fruit Flesh during Fruit Development. In the early developmental stages, the fruit flesh color of the interspecific inbred lines was yellow, gradually turning to dark orange as the fruit ripened. The fruit flesh of *C. maxima* was bright yellow in the early developmental stages, brighter than the interspecific inbred lines. Mature fruits of *C. maxima* also had yellow flesh. In contrast, the flesh of *C. moschata* remained white from 0 to 12 days DAP and then turned pale green during 24−36 DAP. Thereafter, the distinctive pale orange color of *C. moschata* fruit developed rapidly during late development (48 DAP) with minor variations afterward. The development of the fruit flesh color is shown in Figure 2.

Figure 2. Photographs of squash fruit flesh color at different developmental stages (DAP) used in this study. Bar marks 1 cm.

The fruit flesh color inheritance pattern of these interspecific inbred lines represented the combination between *C. maxima*, which had yellow flesh, and also the orange color from *C. moschata*. Interspecific inbred lines were obtained by using *C. maxima* as female parent and *C. moschata* as male parent. Viable seeds were collected and sown to get F_1 plants. However, we were unable to self-pollinate the F_1 plants because of their resulting male sterility. The F_1 plants were backcrossed to *C. maxima* pollens to produce the BC_1F_1 generation. In the BC_1F_1 generation, some male sterility was still observed. Therefore, the BC_1F_1 plants were backcrossed to *C. maxima* again to produce the BC_2F_1 generation. Five self-pollinations were

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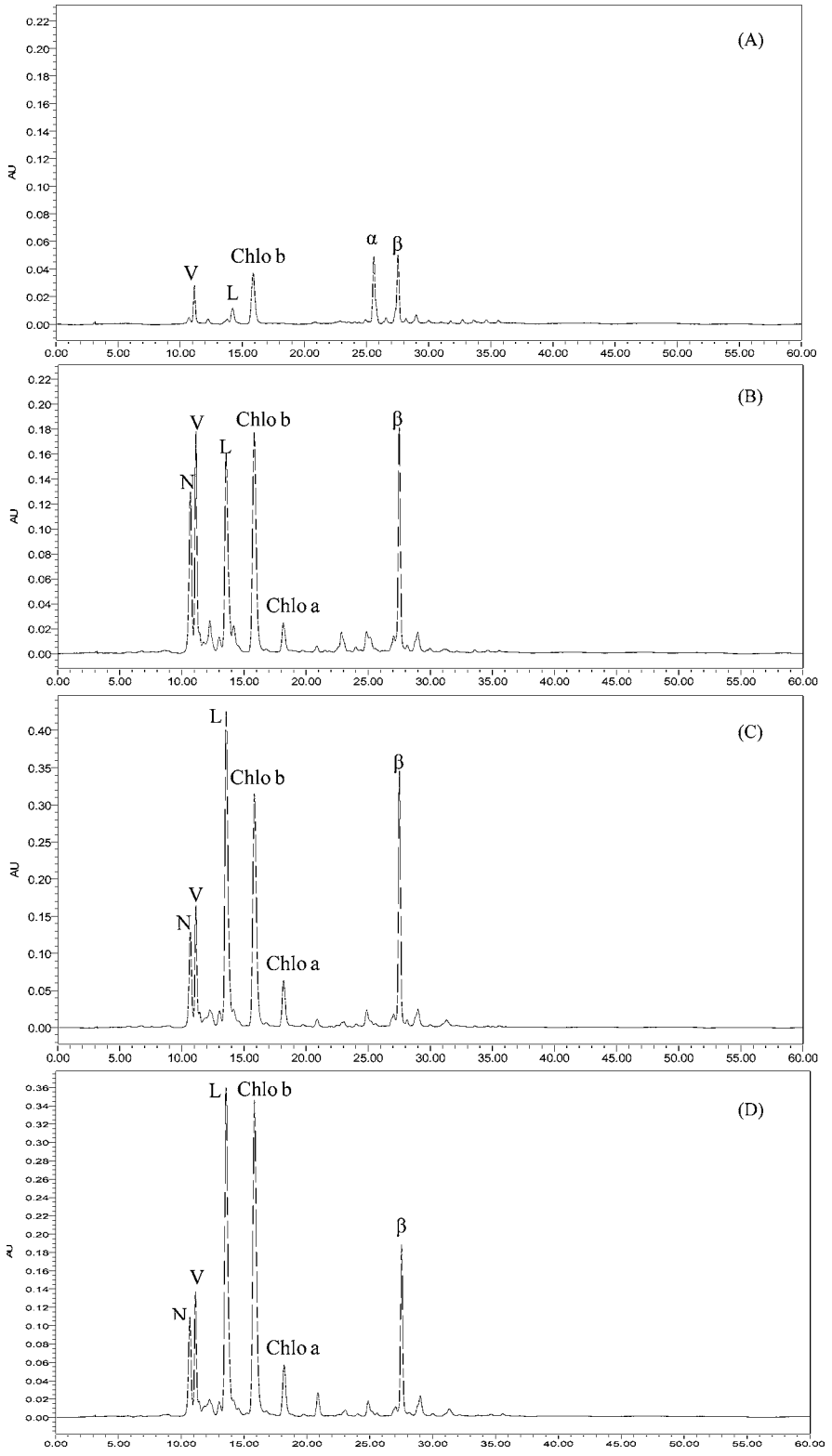

Figure 3. HPLC chromatograms at 450 nm of carotenoids from fruit flesh of squashes at 60 DAP: (A) *C. moschata*; (B) *C. maxima*; (C) Maxchata1; (D) Maxchata2. Peaks: V, violaxanthin; N, neoxanthin; L, lutein; *α*, *α*-carotene; *β*, *β*-carotene; Chlo a, chlorophyll *a*, Chlo b, chlorophyll *b*.

followed subsequently to produce the BC_2F_6 generation (see Supporting Information, Figure S1).

Changes in Carotenoid Composition in the Fruit Flesh of Squashes. There are several previous studies of the carotenoid contents in squash fruits with highly variable results. HPLC analysis was used to evaluate the variation between accumulations of the individual carotenoids during fruit development in different *Cucurbita* species. The HPLC chromatograms of the carotenoid extracts from mature fruits of four squashes are compared in Figure 3. In squashes, the linear carotenoids such as phytoene and lycopene were absent or present in only trace amounts (<0.2 μg/g). The majority of

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the carotenoids detected in squashes were lutein, α-carotene, β-carotene, violaxanthin, and neoxanthin, and the total carotenoid levels of a sample were calculated as the sum of these representative carotenoids. Quantitative differences were detected between squashes studied. It was noticeable that the total carotenoid contents of mature fruit of *C. maxima* and the two interspecific inbred lines were much higher than in *C. moschata* (Figure 4). The interspecific inbred lines had the highest total carotenoid contents, 110.20 and 81.51 μg/g for Maxchata1 and Maxchata2, respectively. *C. maxima* had a total carotenoid content of 74.70 μg/g, whereas *C. moschata* had the lowest total carotenoid with only 24.31 μg/g at fruit ripening.

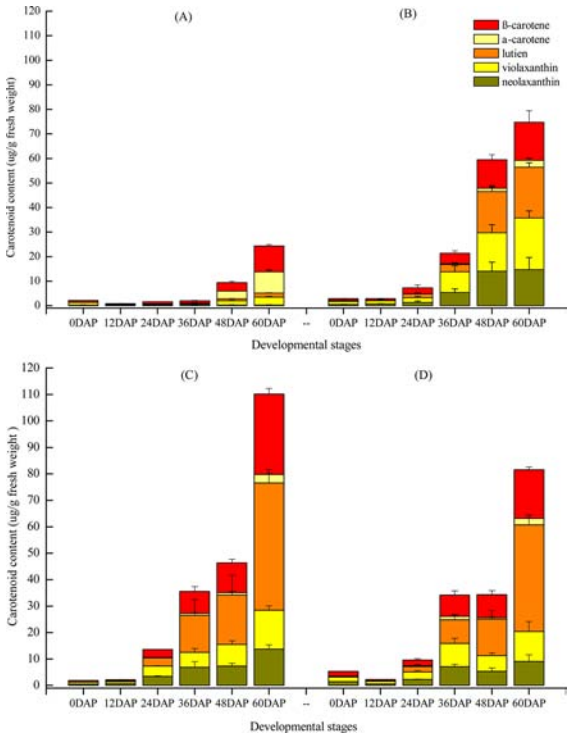

Figure 4. Changes in carotenoid composition during squash fruit development: (A) *C. moschata*; (B) *C. maxima*; (C) Maxchata1; (D) Maxchata2. Values are the mean ± SD of three replications.

Squash varieties with high carotene contents have an orange flesh, whereas varieties with high lutein and low carotene contents show a bright yellow flesh color,[27] suggesting that the colors of squash fruits are related to the levels of individual carotenoids accumulated in fruit. In our study, the major carotenoids in *C. maxima* are violaxanthin, 28.13% (21.01 μg/g), and lutein, 27.66% (20.66 μg/g), followed by β-carotene, 20.72% (15.48 μg/g), and neoxanthin, 19.71% (14.72 μg/g). Yellow flesh phenotype of *C. maxima* appeared to be correlated with violaxanthin and lutein, which are yellow and yellow-orange pigments, respectively. The interspecific inbred lines mainly accumulated lutein and β-carotene. The percentages of lutein in Maxchata1 and Maxchata2 were 43.63% (48.08 μg/g) and 49.54% (40.38 μg/g), whereas percentages of β-carotene were 27.65% (30.47 μg/g) and 22.54% (18.37 μg/g), respectively. The fruit flesh of interspecific inbred lines is orange in color due to the massive amounts of lutein and substantial quantities of β-carotene accumulated. These results are in accordance with the previous study,[28] which showed that Tetsuka Buto, a hybrid of *C. maxima* and *C. moschata*, had a high lutein content of about 56.6 μg/g and a β-carotene content of 30.5 μg/g. In our interspecific inbred lines, the fruit flesh of Maxchata1 was deep orange in color due to a higher total carotenoid content, especially β-carotene, which was almost twice as high as in Maxchata2.

The mature fruits of *C. maxima*, Maxchata1, and Maxchata2 contained only trace amounts of α-carotene: 2.84, 3.22, and 2.38 μg/g, respectively. However, *C. moschata* fruits contained increased α-carotene levels, especially at 48 DAP. This species was the only one to contain α-carotene in mature fruit. In *C. moschata*, α-carotene, 35.58% (8.65 μg/g), and β-carotene, 43.27% (10.52 μg/g), were the most abundant carotenoids. This result corresponded to the pale orange color of the fruit flesh of *C. moschata*, the pale appearance being due to the low concentrations of these compounds in the mature fruit. In particular, the α-carotene and β-carotene contents of *C. moschata* increased later, especially by 48 DAP, corresponding with the pale orange color of the late fruit flesh. In our study, the α-carotene and β-carotene contents of *C. moschata* in the mature fruit were lower than the results obtained by Azevedo-Meleiro and Rodriguez-Amaya.[28] Clearly, different cultivars within the same species of squash can show variation in carotenoid levels and composition.[27]

Different patterns of individual carotenoids accumulation in *Cucurbita* species were observed. Between 0 and 12 DAP of development, all of the carotenoids monitored were either absent or detected only in trace amounts in all squashes studied. The interspecific inbred lines had slightly elevated levels of β-carotene, violaxanthin, and neoxanthin from 24 DAP to the ripening stage, although between 48 and 60 DAP β-carotene contents increased markedly: about 2-fold from 11.20 to 30.47 μg/g and from 8.74 to 18.37 μg/g in Maxchata1 and Maxchata2, respectively. In contrast, the lutein content of the interspecific inbred lines increased rapidly from 24 to 48 DAP and then increased dramatically at 60 DAP from 18.67 to 48.08 μg/g in Maxchata1 and from 13.82 to 40.38 μg/g in Maxchata2. In *C. maxima*, lutein, β-carotene, violaxanthin, and neoxanthin levels increased continuously throughout fruit development. In *C. maxima* and the interspecific inbred lines, α-carotene levels remained low throughout fruit development. *C. moschata* exhibited different patterns of carotenoid accumulation. All of the carotenoids monitored were detected only in small amounts from the beginning of fruit development up to 36 DAP; subsequently, violaxanthin, α-carotene, and β-carotene increased from 48 DAP until the final stage of fruit ripening. Neoxanthin and lutein levels did not show any variation during fruit development in *C. moschata* and were present at only 0.30 and 1.66 μg/g in the mature fruits.

Carotenogenic Gene Expression Profiles during Squash Fruit Development. The accumulation of carotenoids in the fruits of squashes increased during fruit development, especially in the final stages. During fruit maturation, the variety and quantity of carotenoids in fruits increased dramatically, parallel with the acceleration of carotenoid biosynthesis. The variations in expression of carotenogenic genes can explain some of the individual carotenoid accumulation patterns in these species.

The relative transcription levels of the genes encoding for carotenoid biosynthesis were analyzed by qRT-PCR during squash fruit development (Figure 5). The gene-specific primers used for the qPCR analysis were designed on the basis of the corresponding gene sequences (Table1). In *C. moschata*, the comparative transcription levels of all the detected genes were

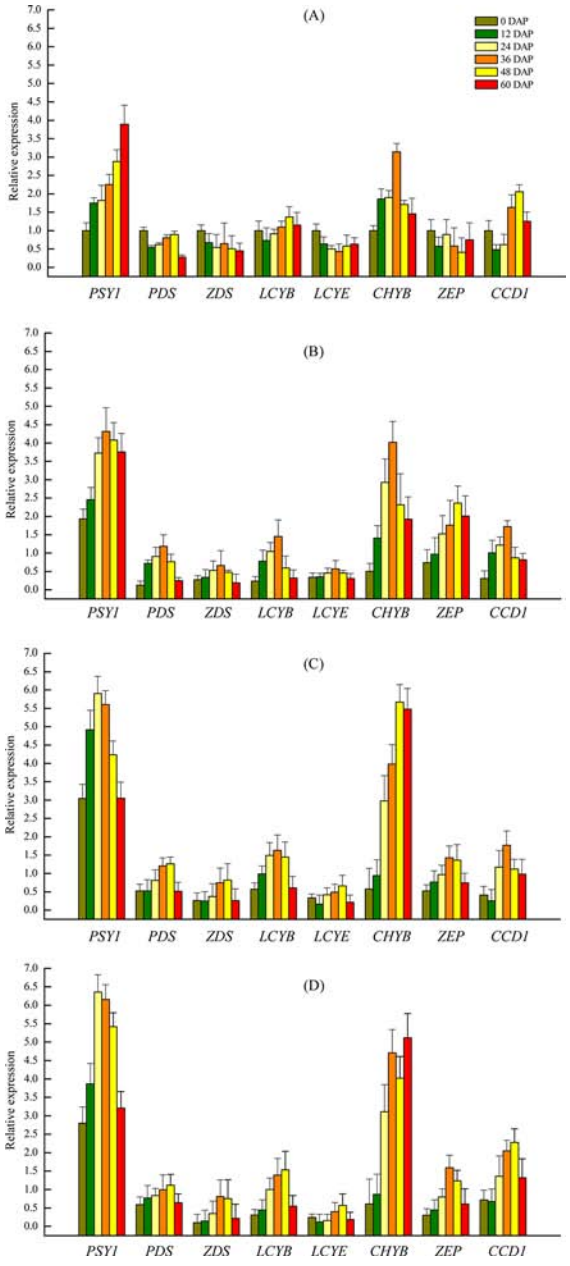

Figure 5. Transcription levels of eight carotenogenic genes in squashes during fruit development: (A) *C. moschata*; (B) *C. maxima*; (C) Maxchata1; (D) Maxchata2. Each bar represents the mean ± SD of three replications.

generally much lower than in the other species. The two interspecific inbred lines showed a similar pattern in the expression of all the genes examined.

The relative expression of *PSY1* in the interspecific inbred lines dramatically increased during early development, reached a peak at 24 DAP, and subsequently decreased until fruit ripening. However, gene expression gradually increased during early fruit development in *C. maxima*, hitting a peak at 36 DAP before slowly decreasing until fruit maturation. In contrast, the *PSY1* gene expressed differently in *C. moschata* and remained up-regulated throughout fruit development, although its transcription level was much lower than in the other squashes. In some plant species, the activity of the *PSY* gene is a rate-limiting step in carotenoid biosynthesis,[35] and it is able to elevate carotenoid accumulation. In tomato, the increase in phytoene synthase activity at any stage of ripening has been paralleled by an increase in *PSY* expression, suggesting that this enzyme is the pacemaker enzyme in carotenoid synthesis in tomato ripening and is regulated at the level of transcription.[36] In addition, transgenic *Psy-1* tomato has shown the important role of *PSY* gene in feed-forward regulation of carotenoid pathway. The expressions of *Psy-1*, *Psy-2*, and *CYC-B* were up-regulated as was the enzyme activity of phytoene synthase and lycopene cyclase B. The increase in phytoene synthase activity can account in part for the elevated level of carotenoids (primarily β-carotene and lutein) as seen in the *Psy-1* mature green fruit.[37] In potato, high transcript levels of *PSY* at early stages of tuberization were observed in the high-carotenoid accession compared to the low-carotenoid accession.[19] In addition, high phytoene synthase has also been associated with β-carotene accumulation in orange carrot roots.[38] Over-expression of the phytoene synthase gene caused carotenoid crystal formation in nongreen tissues of *Arabidopsis*. The sequestration of carotenoid into crystal appeared to be driven by the overexpression of *AtPSY* without requiring additional components such as chloroplast or chromoplast.[38] Metabolic engineering to increase the β-carotene in rice endosperm indicated that unexpected transgenic rice, with only the *PSY* gene, can activate β-carotene synthesis and produce xanthophylls. Thus, the *LCYB* gene was not necessary to achieve β-carotene synthesis in this transgenic rice.[39] As expected, the expression of the *PSY1* gene in squashes was correlated with the amount of carotenoids present, suggesting that *PSY1* activity has also been an important determinant of final carotenoid concentration in ripe fruit. *C. moschata* showed lower expression levels of the *PSY1* gene compared with other squashes, the lack of a carotenoid precursor perhaps explains the low levels of carotenoid accumulation in this species. It has been suggested that a lack of phytoene, which is the requisite substrate for further carotenoid synthesis, could limit the carotenoid pathway in white carrot roots.[40] One possible explanation is that a deficiency in carotenoid precursors results in a low metabolism flux through the pathway, and this might hinder the stimulation of key enzymes in that pathway.[11] The *PSY* knockout tomato mutant confirmed that a lack of phytoene led to the absence of carotenoid accumulation (lycopene and β-catotene) in ripe fruit of the mutant without altering expression levels for the genes involved in the carotenoid pathway.[41]

Fruit ripening of interspecific inbred lines was accompanied by high lutein contents, consistent with very low levels of accumulated α-carotene. In yellow carrot roots, high expression of the *LCYE* gene might be consistent with the accumulation of lutein.[18] Surprisingly, the *LCYE* gene exhibited low expression during fruit development, whereas the high expression of *CHYB* in the interspecific inbred lines was observed in our study. The *CHYB* gene expression pattern in *C. moschata* and *C. maxima* showed a similar pattern, increasing to a peak at 36 DAP and then decreasing dramatically until fruit maturation. In the interspecific inbred lines, all of the genes detected increased during the early developmental stages and subsequently decreased until fruit maturation, with the exception of *CHYB*, which showed higher expression at the ripening stage, and then fell at 48 DAP in Maxchata2. It seems plausible that *CHYB* gene might have a role of lutein accumulation in squashes. It is suggested that other enzymes are also associated with lutein synthesis, such as β- and ε-ring carotene cyclase as well as the carotene hydroxylases.[42] Transformation of *CrtH1* gene

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(*CHYB*) from *Adonis aestivalis* in *Arabidopsis* wild type and a mutant deficient for endogenous β-carotene hydroxylase (*b1b2*) resulted in enhanced accumulation of violaxanthin and lutein. Expression of *CrtH1* in wild type caused a 44−64% increase in lutein level (except for two transgenic lines). In addition, *b1b2* mutant lines showed increase in lutein level ranging from 14 to 72% as compared to *b1b2* mutant.[43] This observation indicates that *CHYB* might contribute to the production of lutein. However, the genetic analysis of carotene hydroxylases revealed the complexity and indicated overlapping function within lutein and zeaxanthin biosynthesis.[44] The principal difference between the two interspecific inbred lines was much higher *CCD1* gene transcription levels in Maxchata2 during later stage of fruit development, similar to *C. moschata*. The transcription level and expression pattern of *CCD1* were similar in *C. maxima* and Maxchata1.

Transcript levels of *ZEP* genes showed no significant difference among squashes. It has been suggested that zeaxanthin, which is synthesized from β-carotene, cannot accumulate because it is epoxidized to violaxanthin by the *ZEP* gene.[28] Therefore, the accumulation of violaxanthin and neoxanthin might be explained in relation to the *ZEP* transcription levels. In *C. moschata*, the steady-state level of *ZEP* transcription was comparatively low, possibly consistent with the lack of violaxanthin and neoxanthin at fruit ripening. On the contrary, the *ZEP* gene expression of *C. maxima* and the interspecific inbred lines increased continuously until fruit ripening, perhaps leading to the accumulation of violaxanthin and neoxanthin. We consider that the results focusing on the differential proportion of *ZEP* and *CHYB* transcription level between Maxchata and *C. maxima* might be the possible explanation for lutein and violaxanthin accumulation. The accumulation of violaxanthin and lutein in *C. maxima*, therefore, accompanied the increase in transcription levels of the *ZEP* genes and the decrease in *CHYB* during ripening. The proportion of *ZEP* and *CHYB* activity might therefore be the result of almost equal amounts of violaxanthin and lutein concentration in *C. maxima* when fruits mature. Conversely, the expression of *ZEP* was lower compared to the *CHYB* gene in both interspecific inbred lines throughout fruit ripening, correlating with the higher concentration of lutein than of violaxanthin and neoxanthin.

PDS, *ZDS*, and *LCYE* have low transcription levels compared to other genes in the carotenoid pathway and were stable throughout fruit development in all squashes. The transcription level of *LCYB* did not differ significantly between the squashes studied. In *C. moschata*, the expression level of *LCYB* was stable throughout fruit development. *C. maxima* and the interspecific inbred lines showed similar patterns of *LCYB* gene expression, which increased continuously from the early stages to peak during the middle of fruit development at 36 or 48 DAP before decreasing until the fruit was ripe. It is difficult to explain the β-carotene accumulation simply from the pattern of expression of the genes leading to its synthesis that were studied here. It is possible that the β-carotene levels in these squash species were not sufficiently different to reflect their transcription levels. Other genes involved in carotenoid biosynthesis as well as other regulatory mechanisms would need to be assessed to explain the differential accumulation of β-carotene in squash. Two genes encoding lycopene β-cyclase have been reported in other carotenogenic fruits; one was a chloroplast-specific lycopene cyclase (*LCYB*), and the other was a chromoplast-specific lycopene cyclase known as *CYCB*. The expression of *CYCB* gene promotes the β-carotene production in several fruits such as tomato[45] and loquat.[46] Therefore, *CYCB* gene might have some contribution to additional carotenoid accumulation in these interspecific inbred lines. Another possible mechanism for differential carotenoid accumulation in these squashes might be due to the differential plastid biogenesis. The *Or* gene was discovered in cauliflower and conferred an orange pigmentation with accumulation of β-carotene in the curd of *Or* mutant.[47] However, the expression of carotenoid biosynthetic genes in *OR* mutant was unaffected. It was concluded that the *Or* gene was not involved in carotenoid biosynthesis but rather created a metabolic sink for carotenoid by inducing the formation of chromoplasts. In addition, the *Or* mutants exhibited an arrest in plastid division so that only one or two chromoplasts were present in the affected cells.[48]

However, we are still far from understanding the overall regulation of carotenoid accumulation in squash. Because metabolic regulation may also occur at other levels, future studies on the protein expression and post-translational control, as well as the potential role of allelic differences and plastid biogenesis, will probably be useful in understanding carotenoid accumulation in *Cucurbita* species.

This study presents the transcriptional expression patterns of carotenogenic genes in relation to different carotenoid composition during fruit development in *Cucurbita* species. The carotenoid content of *C. moschata* at fruit maturation and the expression of all the genes detected was low compared to those in other squashes, especially the *PSY1* gene, as would be expected in a fruit with a pale orange color flesh. The relationship between *CHYB* and *ZEP* gene expression might contribute to the accumulation of violaxanthin and lutein in *C. maxima*, which has yellow mature fruit flesh. The combination of lutein and β-carotene might lead to an orange flesh color in interspecific inbred lines. The high transcription level of *PSY1* gene at earlier stages of fruit development might help elevate the total carotenoid levels in Maxchata. The expression of the *CHYB* gene in the two interspecific inbred lines was higher than in their parents and might be correlated with their high lutein content. Nevertheless, it is difficult to explain the β-carotene accumulation solely on the basis of gene expression. To better understand β-carotene and other carotenoid accumulation in squashes, further research related to translational or post-translational control and plastid biogenesis is required.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table containing genes encoding carotenoiod enzymes isolated from *C. moschata* (Table S1) and figure containing approaches of interspecific hybridization (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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