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Identification, cloning, and expression analysis of three phytoene synthase genes from *Cucurbita pepo*

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Abstract

An essential step in the carotenoid biosynthesis pathway is the formation of phytoene by phytoene synthase (PSY). In this study, three new genes coding PSYs (*CpPSYA*, *CpPSYB*, and *CpPSYC*) were cloned from *Cucurbita pepo* and their expression patterns analysed in three cultivars of summer squash which had a different carotenoid content. The gene sequences had a high similarity with those from other plant species, and their predicted proteins were significantly different from each other. A phylogenetic analysis indicates that CpPSYA and CpPSYB shared a high homology and were also homologous with PSYs from others cucurbits, whereas CpPSYC was more closely related to orthologues from strawberry and carrot. An expression analysis revealed that *CpPSYA* had a higher expression in flowers compared to leaves and showed a differential expression during fruit development. The amount of *CpPSYA* transcript was higher in fruits with a higher carotenoid content than in those with a lower carotenoid content. However, *CpPSYB* and *CpPSYC* showed a relatively high expression in leaves, and their expression in fruits varied among the different cultivars and fruit tissues. These results suggest that the *CpPSY* genes were under different regulatory mechanisms and they may have different roles in *C. pepo*.

Additional key words: fruit development, phylogenetic tree, summer squash, transcriptional analysis.

Introduction

Carotenoids in leaves are parts of light harvesting complexes and protect the photosynthetic apparatus against photo-oxidative damage, whereas in flowers and fruits, they attract pollinators and secure seed dispersal (Bartley and Scolnik 1995, Demmig-Adams *et al.* 1996). They also act as precursors for abscisic acid (ABA) and strigolactones (DellaPenna and Pogson 2006, Gong *et al.* 2012). Carotenoid compounds in the diet are beneficial to human health as antioxidants and vitamin A precursors (Fraser and Bramley 2004).

The plant carotenoid biosynthetic pathway is localized in plastids and the genes involved in this pathway have been well studied (Bartley and Scolnik 1995, Cunningham and Gantt 1998, Hirschberg 2001). There are various regulatory mechanisms controlling the flux of metabolites through the pathway, and phytoene synthase (PSY) play a critical role in this process (Rodriguez-Villalon *et al.* 2009). PSY catalyses the first committed step in the carotenoid biosynthesis through condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to phytoene (Cazzonelli and Pogson 2010). In *Arabidopsis*, a single *PSY* gene is present to regulate this first step in all tissues. However, in other species, this regulatory step is controlled by multiple *PSYs*. In tomato, a model fruit used for the study of carotenoids, three *PSYs* have been described, where *PSY1* is predominant in colored tissues like mature fruits or flowers, whereas *PSY2* is expressed mainly in green

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Abbreviations: DAP - days after pollination; PSY - phytoene synthase; RT-PCR - reverse transcription - polymerase chain reaction; RT-qPCR - real time quantitative polymerase chain reaction.
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tissues (Fraser *et al.* 1999, Giorio *et al.* 2008, Fantini *et al.* 2013), and *PSY3* is a low expressed gene. In maize, there are also three *PSY*s (Li *et al.* 2009). Moreover, recent studies in wheat and *Brassica napus* have found a high polymorphism among *PSY* genes in these species (Cardenas *et al.* 2012, Ravel *et al.* 2013). Within *Cucurbitaceae* family, two *PSYs* with different expression patterns in different tissues and during fruit development have been described in *Cucumis melo* (Qin *et al.* 2011).

The *PSY* gene expression influences carotenoid accumulation in different plant species. For example, during tomato fruit maturation, an increase in *PSY* transcription leads to an increased accumulation of lycopene (Bramley 2002). Similarly, the *PSY1* transcription in satsuma mandarin correlates with carotenoid accumulation during fruit maturation (Ikoma *et al.* 2001). Also, transgenic tobacco and potato plants over-expressing *PSY* have increased carotenoid concentration whereas in rice, which does not contain any carotenoids in the endosperm, genetic engineering with *PSY* contributed to the accumulation of β-carotene (Ye *et al.* 2000, Paine *et al.* 2005). It has been shown in cassava that a minor genetic alteration causing an allelic polymorphism in *PSY2* can affect the catalytic efficiency of the enzyme, resulting in an increased carotenoid accumulation in the root (Welsch *et al.* 2010). In addition, association mapping and a linkage analysis in pepper or wheat show that *PSY* genes are linked to QTLs for carotenoid content (Huh *et al.* 2001, Howitt *et al.* 2009). Considering these important roles, *PSY* is a preferred gene candidate for understanding the molecular control of carotenoid accumulation in most species.

Based on our previous study on summer squash (Obrero *et al*. 2013), we examined the *PSY* gene expression in three cultivars of *Cucurbita pepo* which had a different carotenoid content. A preliminary investigation of the expression of *PSY* suggests the possibility of multiple enzymes in summer squash since despite a high content of carotenoids is in flowers the amount of *PSY* transcripts is low (Obrero *et al.* 2013).

Materials and methods

Plants: Three cultivars of *Cucurbita pepo* L. were selected on the basis of their contrasting fruit peel colour and carotenoids content (Fig. 1): cv. Scallop from *C. pepo* ssp. *ovifera* with white fruits and a low content of carotenoids in skin and flesh, cv. MU_CU16 from *C. pepo* ssp. *pepo* with green fruits and a high carotenoids content in skin but low in flesh, and cv. Parador from *C. pepo* ssp. *pepo* with yellow-orange fruits, with a moderate carotenoids content in skin and flesh (Fig. 2). Experiments were conducted from December 2010 to July 2011 under standard greenhouse conditions in the station La Mojonera, Almería, Spain. Tissue samples, pooled from three biological replicates, for each cultivar were collected from leaves, flowers at two stages of development (pre-anthesis and anthesis), ovaries, and fruits (skin and flesh) at four stages of development (3, 5, 7, and 20 days after pollination, DAP).

Cloning *CpPSYA*, *CpPSYB, and CpPSYC*: A total RNA was extracted using a *TRIsure* reagent (*Bioline*, London, UK) according to the manufacturer´s instructions. The extracted RNA from leaves, flowers, and fruits was treated with *DNAse I* (*Life Technologies*, Carlsbad, CA, USA) to eliminate a genomic DNA present in the samples. cDNA was synthesized using *Superscript III* reverse transcriptase (*Life Technologies*). Specific forward and reverse primers were designed in the conserved regions of *C. pepo PSY* clones from the *Cucurbit Genomics* database:
F5OTW9201BSUAE, F5OTW9201C5BW2, F5OTW9201E4A5U, F5OTW9202HWIYN, F5OTW9202F83X0, F5OTW9202IFJ12, F5OTW9202GUGZ4, F5OTW9202GTPNY, F5OTW9201D33AL, F5OTW9202IGIWL, F5OTW9202IRKWJ, F5OTW9201CZU1X.

The 3' end cDNA sequences of *CpPSY* genes were obtained using a *GeneRacer* kit (*Life Technologies*). The 5' end cDNA sequences were obtained using the 5′-*RACE* system for a rapid amplification of cDNA ends *v. 2.0* (*Life Technologies*). All of the cloning reactions were performed using *Platinum®Taq* DNA polymerase of a high fidelity, and PCR products were cloned into a *TOPO XL* PCR cloning kit (*Life Technologies*) and sequenced. Primers used are included in Table 1.

Gene expression analysis: A total RNA was extracted using a *TRIsure* reagent (*Bioline*) according to the manufacturer´s instructions. The RNA integrity was assessed by a microcapillary electrophoresis with an *Experion RNA StdSens* chip and an *Experion* bioanalyzer (*Bio-Rad Laboratories*, Hercules, CA, USA). All RNA samples showed RNA quality indicator (RQI) values higher than nine. cDNA was synthesized from 1 μg of total RNA for each sample using a *QuantiTec* reverse transcription kit (*Qiagen*, Hilden, Germany) with a blend of oligo-dT and random primers according to the manufacturer's instructions as described previously (Obrero *et al*. 2013). Primer pairs for RT-qPCR amplification were designed using the *PRIMER3* software (Rozen and Skaletsky 2000). The RT-qPCR analysis was performed using a *LightCycler 1.5* system (*Roche*, Penzberg, Germany). All reactions were performed using a *SYBR Green* master mix (*Roche*) under the same conditions as described by Obrero *et al*. (2013). Each reaction sample was prepared in four technical replicates with a negative control using water as template. Raw data were analysed using the *LightCycler* software *v. 4.* The expressions of the target genes were calculated

using an advanced relative quantification model with an efficiency correction, a multiple reference gene normalization, and the use of error propagation rules (Hellemans *et al.* 2007). Based on previous results, *Elongation factor 1-alpha* (*EF1A*) and *Protein phosphatase 2A* (*PP2A*) were selected as reference genes for a multiple gene normalization (Obrero *et al.* 2011).

Bioinformatics analysis: The nucleotide sequence and multiple sequence alignments were performed by *Geneious Pro 5.5.6* (http://www.geneious.com). An additional homology analysis was conducted using *BLAST* tool (http://www.ncbi.nlm.nih.gov/BLAST/) and *Cucurbit Genomics Database* (http://www.icugi.org/). A phylogenetic tree was generated using the neighbor-joining method included in the *CLUSTALW* program and a dendogram was constructed by the *MEGA5.1* program (Tamura *et al.* 2011). The analysis was carried out from a conserved domain Trans_IPPS_HHB (from 138 to 415 amino acids). The bootstrap analysis of the neighbor-joining tree was performed using 1 000 replicates.

Fig. 1. Leaves, flowers at anthesis, flowers before anthesis and ovaries, and fruits of three cultivars of *Cucurbita pepo*: Scallop (*left*), MU_CU16 (*middle*), and Parador (*right*). The *scale bar* represents 1 cm.

Results

Three members of *PSY* gene family (Cp*PSYA*, *CpPSYB*, and *CpPSYC*) were isolated from two subspecies of *C. pepo* ssp. *ovifera* (GenBank accession Nos. JX912284, JX912281, and JX912286) and *C. pepo* ssp. *pepo* (acc. Nos. JX912285, JX912282, and JX912287). The alignment of *PSY* DNA fragments amplified by 3' RACE clearly showed at least three *PSY* genes present in *C. pepo* (Fig. 3*A*). The *CpPSYA* cDNA sequences obtained were 1 621 and 1 599 bp for the ssp. *ovifera* and *pepo*, respectively. These included a 271 bp

Table 1. Primers used for analyses.

Primer	Sequence (5´to 3´)	Use
GeneRacer 3' primer	GCTGTCAACGATACGCTACGTAACG	3´RACE PCR
GeneRacer 3' nested primer	CGCTACGTAACGGCATGACAGTG	
PSY_F 3' specific primer	GAGATGTTGGAGAAGATGCTAG	
PSY_F 3' nested primer	GATGCTAGAAGAGGAAGA	
AAP 5´ primer	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	5´RACE PCR
AUAP 5´ primer	GGCCACGCGTCGACTAGTAC	
PSY_R 5´ specific primer	AGAAATCCGAAGCTCGATAGGG	
PSY_R 5´ nested primer	CGTTAGGAAGAGACAACCAACG	
PSYA_F full length	CACCCTTTTACACGATCAAACA	full-length
PSYA_R full length	GTGATGTGAGTTCATTAAGGGCTA	
PSYB_F full length	AGTGGCCTCAACTCAAATGC	
PSYB_R full length	GCCATTGGTAGTGCCATCAT	
PSYC_F full length	GGTGGCTGTGAAGAGAAGATGT	
PSYC_R full length	ACAACGAGCGAATCGTGACA	
PSYA_F	GTGGGCTTCGTTGCTATTATATCG	qPCR
PSYA_R	GTGATGTGAGTTCATTAAGGGCTA	
PSYB_F	GTGGGCTTCATTGCTACTATATCGG	
PSYB_R	GCCATTGGTAGTGCCATCAT	
PSYC_F	CCTTTGGAAAAGCTGTGGTG	
PSYC_R	ACAACGAGCGAATCGTGACA	

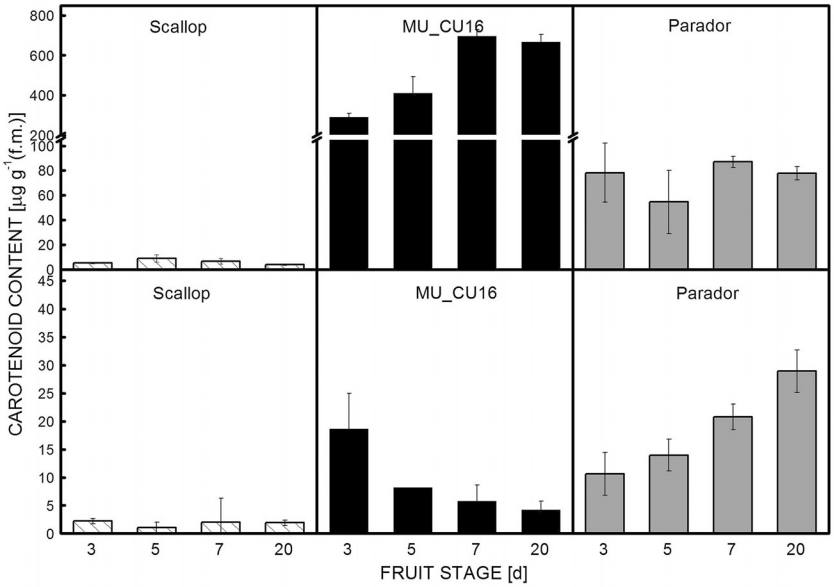

Fig. 2. Carotenoid accumulation in fruit skin (the *top panel*) and flesh (the *lower panel*) by HPLC from three biological replicates of *C. pepo* cvs. Scallop, MU_CU16, and Parador at 3, 5, 7, and 20 DAP.

5' untranslated region (UTR), a 1 260 bp open reading frame (ORF), and 3' UTR of 90 bp and 68 bp for ssp. *ovifera* and *pepo*, respectively. The *CpPSYB* cDNA sequence had 365 bp 5' UTR, ORF of 1236 bp and 63 bp 3´UTR, whereas the *CpPSYC* cDNA sequence was 1173 bp including 18 bp 5´ UTR and 166 bp 3´ UTR. CpPSYA had a deduced protein sequence of 420 amino acids. *BLAST* results show CpPSYA shared the highest homology with chloroplast *PSY* from *Cucurbita moschata* (97 % identity). A deduced protein for CpPSYB had 412 amino acids and shared the highest homology with *PSY* from *Cucumis melo* (89 % identity), whereas CpPSYC had a deduced protein of 391 amino acids and shared the highest homology with *PSY Fragaria* × *ananassa* (80 % identity). The alignment of the three protein sequences showed 280 identical amino acids (65.7 %) with CpPSYA sharing an 89 and 75 % identity with CpPSYB than CpPSYC, respectively. The major differences among the three proteins were present in the N-terminus; CpPSYA and CpPSYB started with the same residues, however, CpPSYC was shorter and had a repeat of nine serines at the start of the protein (Fig. 3*B*). The comparison of the three genes with the genomic sequence of *PSY1* from *Cucumis melo* showed a

strong similarity in structure among all sequences. The first exon of *CpPSYC* gene was shorter than *PSY1* of *C. melo*, *CpPSYA*, and *CpPSYB*; however, the sixth exon was longer in *CpPSYC* than in the rest of the sequences. Comparison of the predicted PSY protein sequences between *C. pepo* ssp. *pepo* and *C. pepo* ssp. *ovifera* showed a 99 % identity for all the three PSYs with only four residues in PSYA, two in PSYB, and three in PSYC that were different between the subspecies. These differences could not explain the differential accumulation of carotenoids in these subspecies.

To understand the relationship between the summer squash PSY protein sequences and PSY protein sequences from a broad range of plants, a phylogenetic tree was constructed (Fig. 4). The analysis shows that our proteins had a high homology to other plant *PSY*s. CpPSYA and CpPSYB shared the same subclades; this group comprised *Cucurbitaceae* family (*Cucurbita pepo*, *Cucumis melo*, and *Cucurbita moschata*). However, CpPSYC protein clustered separately, being closely related to PSY from *Fragaria* × *ananassa* and PSY1 from *Daucus carota.*

Gene expression was examined in flowers and leaves to determine any tissue-specific expression of these three *PSY*s (Fig. 5). *CpPSYA* showed a higher expression in flowers compared with *CpPSYB* and *CpPSYC*, except in

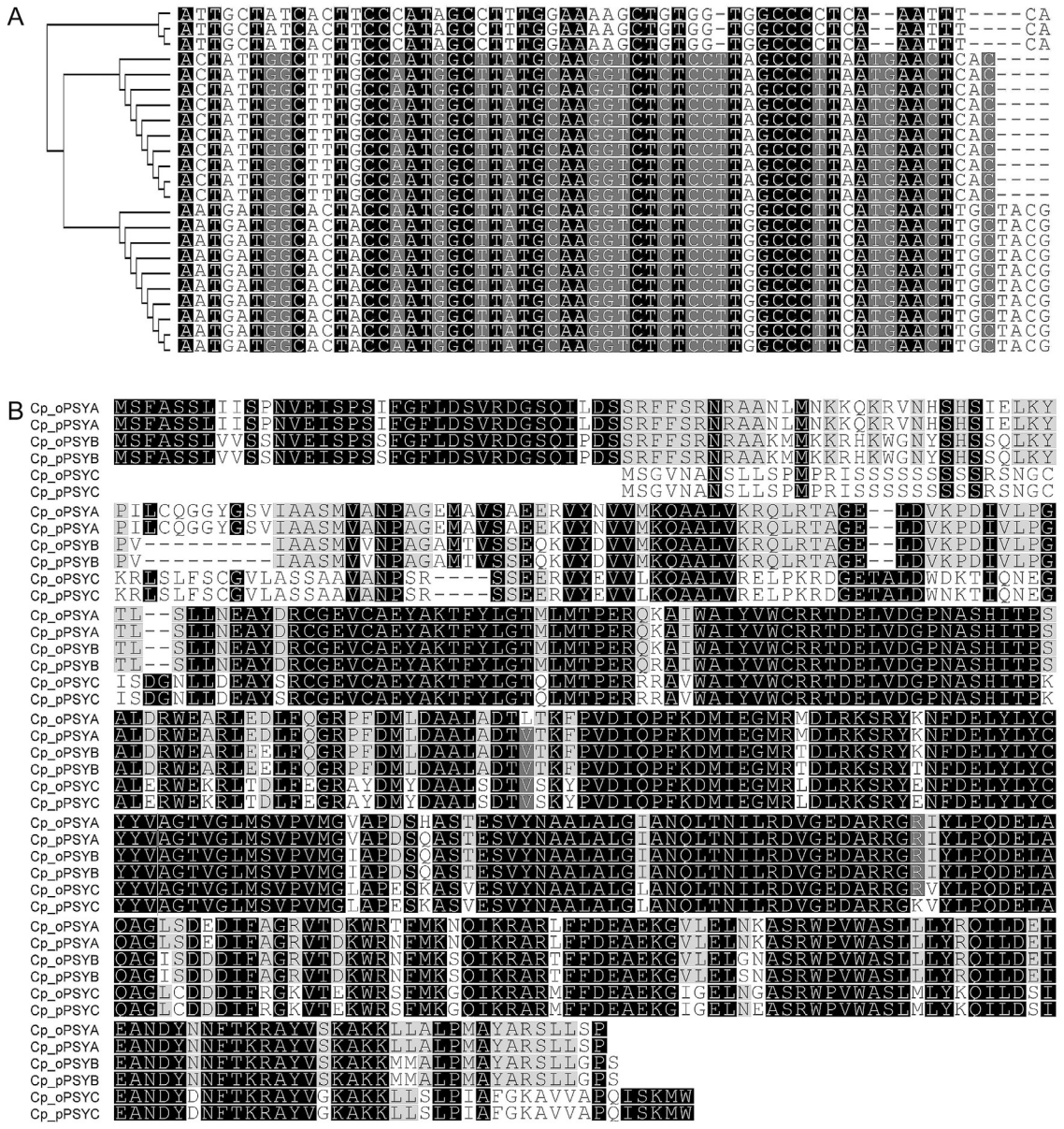

Fig. 3. *A* - The sequence alignment and phylogenetic analysis of 23 *PSY* DNA fragments show the presence of three *PSY* genes in *C. pepo*. *Highlighted* nucleotides show some similarities and differences between the gene sequences. *B* - The sequence alignment of CpPSYA, CpPSYB, and CpPSYC predicted protein sequences from *C. pepo* ssp. *ovifera* (Cp_o) and *C. pepo* ssp. *pepo* (Cp_p). *Black shading* indicate a 100 % similarity and *gray shading* at least a 50 % similarity between the sequences.

cv. Parador, where the *CpPSYC* expression was also high. The transcription of *CpPSYB* was higher in leaves than in flowers and ovaries. The expression of *CpPSYC* was high in leaf and flower tissues, especially in Parador and MU_CU16. On the other hand, the white cultivar Scallop had a lower expression of all the three *CpPSYs* in all the tissues examined, compared to the other cultivars.

We compared the expression patterns of all the three genes in skin and flesh in different fruit stages (Fig. 6). In fruit skin, the *CpPSYA* expression in Parador and MU_CU16 increased during fruit development, being the highest at 20 DAP (in Parador 8.9-fold higher than at 3 DAP). The *CpPSYA* transcripts were barely detectable in Scallop, except for a relative high expression at a 7 DAP stage. In flesh, the *CpPSYA* expression in Parador was similar to that in skin, increasing during fruit development, whereas MU_CU16 showed a high amount of transcripts at 3 and 20 DAP. The relative expressions of *CpPSYA* in flesh at 20 DAP differed significantly among the three cultivars (9-fold and 60-fold higher in Parador than in MU_CU16 and Scallop, respectively). The expression pattern in Scallop flesh was very similar to that in skin.

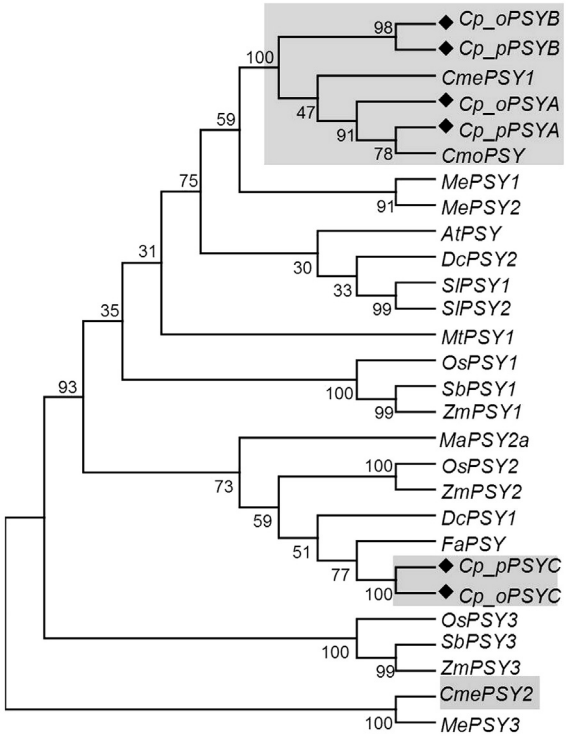

Fig. 4. The phylogenetic tree of CpPSYs (*diamonds*) and other related sequences. Numbers below the branches are the neighbor-joining bootstrap values. PSY sequences from the *Cucurbitaceae* family are indicated by the *shaded* boxes. GenBank accession numbers: (*Arabidopsis*) AtPSY, L25812; (squash) CmoPSY, AEK86564; (melon) CmPSY1, JF745118; CmPSY2, JF745117; (carrot) DcPSY1, DQ192186; DcPSY2, DQ356430; (strawberry) FaPSY, ACR61392; (banana) MaPSY2a, AF33592; (cassava) MePSY1, ACY42664; MePSY2, ACY42665; (rice) OsPSY1, AY445521; OsPSY2, AK073290; OsPSY3, DQ356431; (sorghum) SbPSY1, AY705389; SbPSY3, AY705390; (tomato) SlPSY1, EF534740; SlPSY2, EF534738; (maize) ZmPSY1, AAX13806; ZmPSY2, AAQ91837; ZmPSY3, DQ356430.

The expression pattern for *CpPSYB* in fruit skin was also different in the three cultivars examined. The *CpPSYB* transcriptions were high at 3 and 20 DAP in Parador, at 5 and 7 DAP in MU_CU16, and at 7 and 20 DAP in Scallop. Overall, the *CpPSYB* expression in fruit flesh of the three cultivars was different from that in fruit skin, with a higher expression at a late fruit stage. This was similar to the *CpPSYA* expression pattern in flesh, however, the *CpPSYB* expression increased in all stages compared to *CpPSYA*.

The *CpPSYC* showed an interesting expression pattern in fruit. In the fruit skin of Parador and MU_CU16, the *CpPSYC* expression increased at early stages at 20 DAP, and when the fruit was ripe, there was a significant reduction in expression. In fruit flesh, there was a low *CpPSYC* expression in all the cultivars except for a high expression observed at a 3 DAP stage in MU_CU16 (15.5-fold and 622-fold higher than in Scallop and Parador, respectively).

To understand how the expression of the *PSY* genes might be associated with carotenoid accumulation in fruit tissues, the Pearson's correlation was performed between the total carotenoid content and the relative gene expression in fruit skin and flesh across the time points during development (Table 2). A strong correlation was observed for *CpPSYA* in the skin of MU_CU16 as well as the flesh of Parador. Similarly, *CpPSYB* showed a relationship with the carotenoid accumulation in both the skin and flesh of Parador, albeit not statistically significant ($P > 0.05$). In general, the *CpPSYC* expression was low in comparison with the *CpPSYA* and *CpPSYB* expressions. Nevertheless, the *CpPSYC* expression significantly correlated with the carotenoid accumulation in MU_CU16 fruit flesh, though no such a relationship was

Table 2. Pearson's correlation (r) and probability values for a statistical significance (in parentheses) between the gene expression and the total carotenoid content in fruit tissues from three *C. pepo* cultivars (* indicate a significant correlation at $P \leq 0.05$).

Tissues	Scallop			MU_CU16			Parador		
	CpPSYA	*CpPSYB*	*CpPSYC*	*CpPSYA*	*CpPSYB*	*CpPSYC*	*CpPSYA*	*CpPSYB*	*CpPSYC*
Skin	0.14 (0.43)	-0.08 (0.54)	0.10 (0.45)	0.90 (0.05)*	0.45 (0.27)	-0.27 (0.67)	0.17 (0.41)	0.81 (0.09)	-0.89 (0.05)*
Flesh	0.47 (0.26)	0.62 (0.18)	0.52 (0.24)	0.57 (0.21)	0.21 (0.39)	0.97 (0.01)*	0.88 (0.05)*	0.84 (0.07)	-0.06 (0.52)

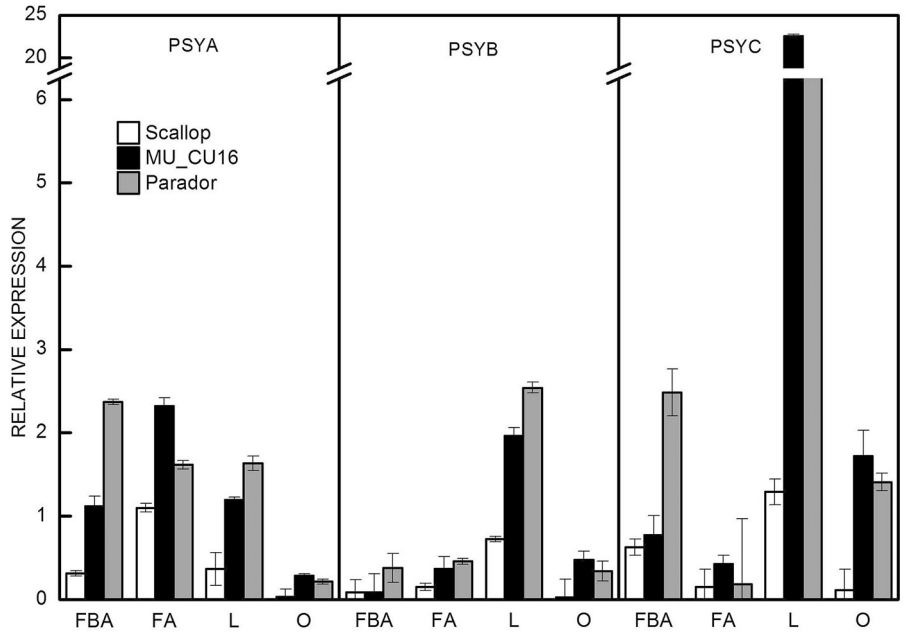

Fig. 5. The relative expression of *CpPSYA*, *CpPSYB*, and *CpPSYC* in different organs of three *C. pepo* cultivars: flowers before anthesis (FBA), flowers at anthesis (FA), leaves (L), and ovaries (O). Data are from three pooled biological replicates with *error bars* representing SE from four technical replicates. The expression of *PP2A* and *EF1A* were used to normalize the mRNA content for each sample.

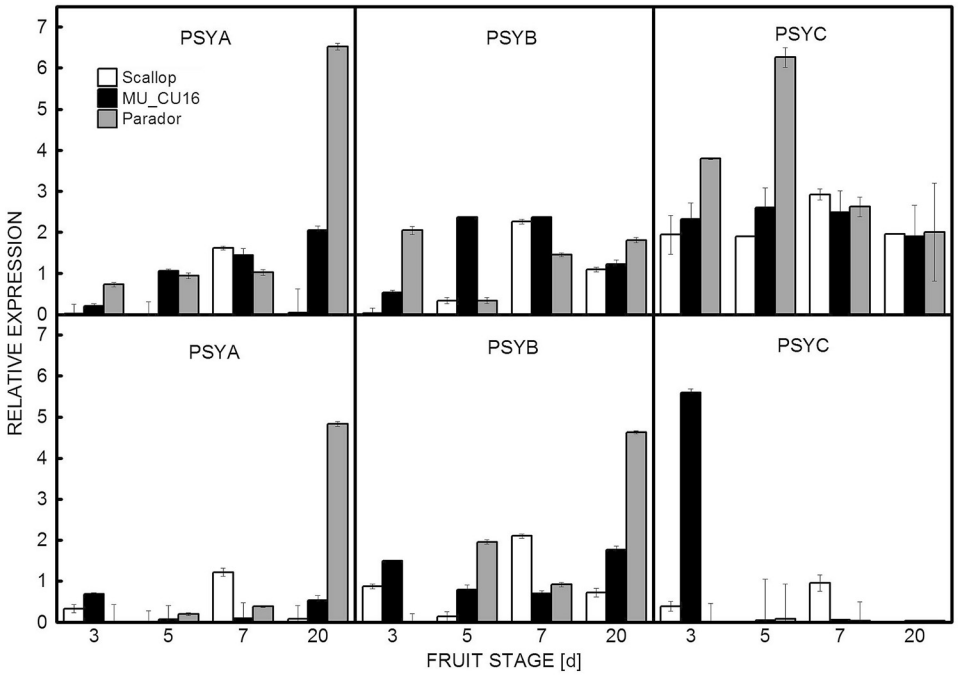

Fig. 6. The relative expression of *CpPSYA*, *CpPSYB*, and *CpPSYC* genes in fruit skin (the *top panel*) and flesh (the *bottom panel*) of three *C. pepo* cultivars during fruit development (3, 5, 7, and 20 DAP). Data are from three pooled biological replicates with *error bars* representing SE from four technical replicates. The expression level of *PP2A* and *EF1A* were used to normalize the mRNA content in each sample.

observed in Scallop or Parador. However in Parador, *CpPSYC* showed a negative correlation with the total carotenoid content in fruit skin. Overall, the correlation analysis suggests that the expressions of the three *PSY*s were important to carotenogenesis in the cultivars examined, and they might have some specificity in controlling carotenoid content in fruit tissues.

Discussion

PSY has a critical role in the carotenoid biosynthesis pathway and therefore plays an important function in the formation of colour in flowers and fruits (Hirschberg 2001, Cazzonelli and Pogson 2010). In some crop species, there are multiple *PSY* genes responsible for this enzyme step suggesting that these genes may have diverse roles (Bartley and Scolnik 1993, Busch *et al*. 2002, Li *et al.* 2008, Arango *et al.* 2010, Qin *et al*. 2011). In this study, we isolated different *PSY* genes present in *C. pepo* using the RACE technique and found at least three *PSY* genes in this species. Both the homology and phylogenetic analysis indicate that these genes had a high degree of conservation. *CpPSYA* and *CpPSYB* were closely related, whereas CpPSYC was more similar to *PSY* from other species. The comparison of the three genes with the *PSY1* gene structure from *Cucumis melo* indicates that these *CpPSY* genes contained six exons and five introns, as previously described in other species (Wang *et al.* 2009, Zhao *et al.* 2011).

The expression analysis of the *CpPSYA* gene revealed a different transcription of *CpPSYA* in flesh and skin between the cultivars with colored and white fruits, especially at maturity. Also, the *CpPSYA* transcription increased during fruit development in the skin of Parador and MU_CU16 and in the flesh of Parador. This is consistent with a significant increase in carotenoid content observed in Parador and MU_CU16 fruit tissues (Obrero *et al*. 2013). In contrast to *CpPSYB* and *CpPSYC*, the transcription of *CpPSYA* in flowers was higher than in leaves. *PSY* expression is known to be highly regulated and shows a tissue-specific expression pattern (Dobrowolska 2006). Several studies have shown that the transcription of *PSY* is regulated developmentally, with an elevated expression in a chromoplast-containing tissue (Fraser *et al.* 2002, Rodrigo *et al.* 2004). At the early stages of fruit development, carotenoids are co-located with chlorophyll within chloroplasts and assist in photosynthesis. However during ripening, chloroplasts are transformed into chromoplasts where carotenoids accumulate to high concentrations. In tomato, *PSY1*, which is expressed in ripening fruits and petals, contributes to phytoene formation in chromoplasts, whereas *PSY2* is expressed in all plant organs with high levels in leaves (Bartley and Scolnik 1993, Giorio *et al*. 2008). Based on the expression pattern, it is conceivable that *CpPSYA* could be preferentially acting in chromoplast-rich tissues.

The expression pattern for the *CpPSYB* gene was similar to the *CpPSYA* in fruit flesh, but appeared to have a less differential expression in fruit skin. The highest *CpPSYC* expression was found in leaves of MU_CU16 and Parador and the lowest in mature fruits of all the cutivars. The *CpPSYC* transcription also showed a significant correlation with the total carotenoid content in the flowers, leaf tissues, as well as flesh of MU_CU16. A recent study in banana cultivars showed that *PSY1* and *PSY2a* mRNA is detected in leaf and green fruit tissues of two cultivars with a low and high carotenoid content, but not in ripe fruits suggesting an increasing degradation of these transcripts at this stage of fruit development (Mlalazi *et al.* 2012). Interestingly, the phylogenetic tree shows that *CpPSYC* is more closely related to *MaPSY2a* (*Musa acuminata*) than are *CpPSYA* and *CpPSYB*.

Given the important role of PSY as the first committed enzyme in the carotenoid pathway, the significant correlations between the gene expression and the carotenoid content that were observed are indications that these *PSY* genes may act as rate-limiting steps, and their expression could be predictive of carotenoid accumulation in summer squash (Li *et al*. 2008, 2009, Arango *et al*. 2010). The strong association between the *PSYA* expression and the total carotenoid accumulation in fruit skin and flesh points to a significant *PSYA* role during carotenogenesis in fruit. In our previous study, a *LCYe* gene was implicated in a transcriptional control of carotenoid accumulation in these fruits, whereas *PSY* transcripts did not show any strong correlation with a carotenoid content due to the presence of multiple genes (Obrero *et al*. 2013). It is possible that both *LCYe* and *PSYA* control important rate-limiting steps during carotenoid accumulation in fruits. Overall, the three *PSY* genes found in *C. pepo* showed unique expression patterns that were related to the carotenoid accumulation pattern observed in the different cultivars. A further analysis of these genes under different environmental conditions would help to further understand their different roles in this species (Li *et al*. 2008, Welsch *et al.* 2008).

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