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ORIGINAL PAPER

Expression Profile of Carotenoid Cleavage Dioxygenase Genes in Summer Squash (*Cucurbita pepo* L.)

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Abstract Carotenoids are important dietary components that can be found in vegetable crops. The accumulation of these compounds in fruit and vegetables is altered by the activity of carotenoid cleavage dioxygenases (CCDs) enzymes that produce their degradation. The aim of this work was to study the possible implication of *CCD* genes in preventing carotenoid storage in the horticultural crop summer squash (*Cucurbita pepo* L.). The relationship between the presence of these compounds and gene expression for *CCD*s was studied in three varieties showing different peel and flesh colour. Expression analysis for the *CCD* genes *CpNCED1*, *CpNCED2*, *CpNCED3*, *CpNCED9*, *CpCCD1*, *CpCCD4a*, *CpCCD4b* and *CpCCD8* was carried out on different organs and at several fruit developmental stages. The results showed that the *CpCCD4a* and *CpCCD4b* genes were highly expressed in the variety with lowest carotenoid content suggesting a putative role in carotenoid accumulation pattern in summer squash fruit.

Keywords Zucchini · Courgette · Carotenoid cleavage dioxygenase · Carotenoid content · qPCR

Abbreviations

ABA	Abscisic acid
CCD	Carotenoid cleavage dioxygenase
NCED	Epoxycarotenoid dioxygenase

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Introduction

Since carotenoids are precursors for vitamin A and present antioxidant activity, but humans cannot synthesise them, these compounds are essential for health [1, 2]. Moreover, these pigments are important in vegetables and fruit not only by its nutritional value but also for producing coloration and acting as precursors for aroma compounds, being the development of new varieties with high carotenoids content of interest [3–5].

Summer squash (*Cucurbita pepo* L.) is an appreciated horticultural crop with a wide range of variety. The fruit is consumed at an immature stage when carotenoids are usually accumulated at lower levels than in the mature fruit. For this reason, the selection of varieties with high carotenoids content, as well as the molecular mechanism controlling this trait, result of interest. With this end, in a previous work the expression profile of the principal genes involved in carotenoid biosynthesis as well as the carotenoid content in contrasting varieties showing different peel and flesh was investigated [6]. From this work it was concluded that transcript levels for some genes like *LCYe* were higher in varieties accumulating carotenoids in the fruit and could be regulating the process. Nevertheless, other regulatory mechanisms at a different level than the main pathway could be also involved. The accumulation of these compounds depends on the storage capacity of carotenoids in cells and their degradation produced by carotenoid cleavage dioxygenases (CCDs) enzymes [7, 8].

The CCD enzymes are involved in carotenoid catabolism, cleaving them and giving rise to apocarotenoids. These compounds are biologically significant including hormones such as abscisic acid (ABA) and strigolactones, other non-volatiles apocarotenoids such as bixin, blumenin or crocetin, as well as flavours and scents. These enzymes

show different carotenoid substrates and cleavage sites and have been classified as CCDs if they metabolize carotenoids to strigolactones and volatile apocarotenoids, or 9-cis-epoxycarotenoid dioxygenases (NCEDs) when they are directly involved in abscisic acid (ABA) synthesis [9–11]. In *Arabidopsis thaliana* nine *CCD* genes have been described as: five of the members are NCEDs (2, 3, 5, 6 and 9) and the other ones are CCDs (1, 4, 7 and 8) [12]. The implication of CCD enzymes on carotenoid accumulation have been studied in several crops such as citrus, watermelon, peach, tomato and potato in which, in general, the transcriptional level of this kind of genes was inversely related with the fruit carotenoid content [13–19].

CCD family in crops of agronomic importance such as summer squash have barely been studied. For this reason, the aim of this work was to test the hypothesis that genes encoding these enzymes could be controlling carotenoid accumulation. Expression profile for several *CCD* genes was studied from different organs and developmental stages of three varieties of summer squash presenting a different fruit carotenoid accumulation pattern. The results described in this work completed those previously obtained about carotenogenic genes [6] and improved our knowledge of possible factors controlling carotenoid accumulation in this plant.

Materials and Methods

Plant Material

Plants of three cultivars with differential carotenoid accumulation in the fruit [6] were used: UPV196 *C. pepo* ssp *ovifera* Scallop (COMAV) with white peel and flesh; MU_CU16 *C. pepo* ssp *pepo* (COMAV) with green peel and nearly white flesh, and cv Parador, *C. pepo* ssp *pepo* (Gautier) with yellow peel and flesh. Leaves, female flowers before and after anthesis, ovary before anthesis as well as fruit at 3, 5, 7 and 20 days after pollination (dap) (Fig. 1) were frozen in liquid nitrogen and kept at −80 °C. The peel or exocarp (E3, E5, E7, E20) and the flesh or mesocarp (M3, M5, M7, M20) were separately sampled.

Nucleic Acid Isolation and cDNA Synthesis

Samples were powdered in liquid nitrogen and genomic DNA as well as total RNA was isolated by using DNAzol (Invitrogen, Carlsbad, CA) or TRIsure (Bioline, London, UK) as reagent respectively, according to the manufacture's protocol. Nucleic acid concentration was quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was tested using the Experion automated electrophoresis system (Bio-Rad, Hercules, CA).

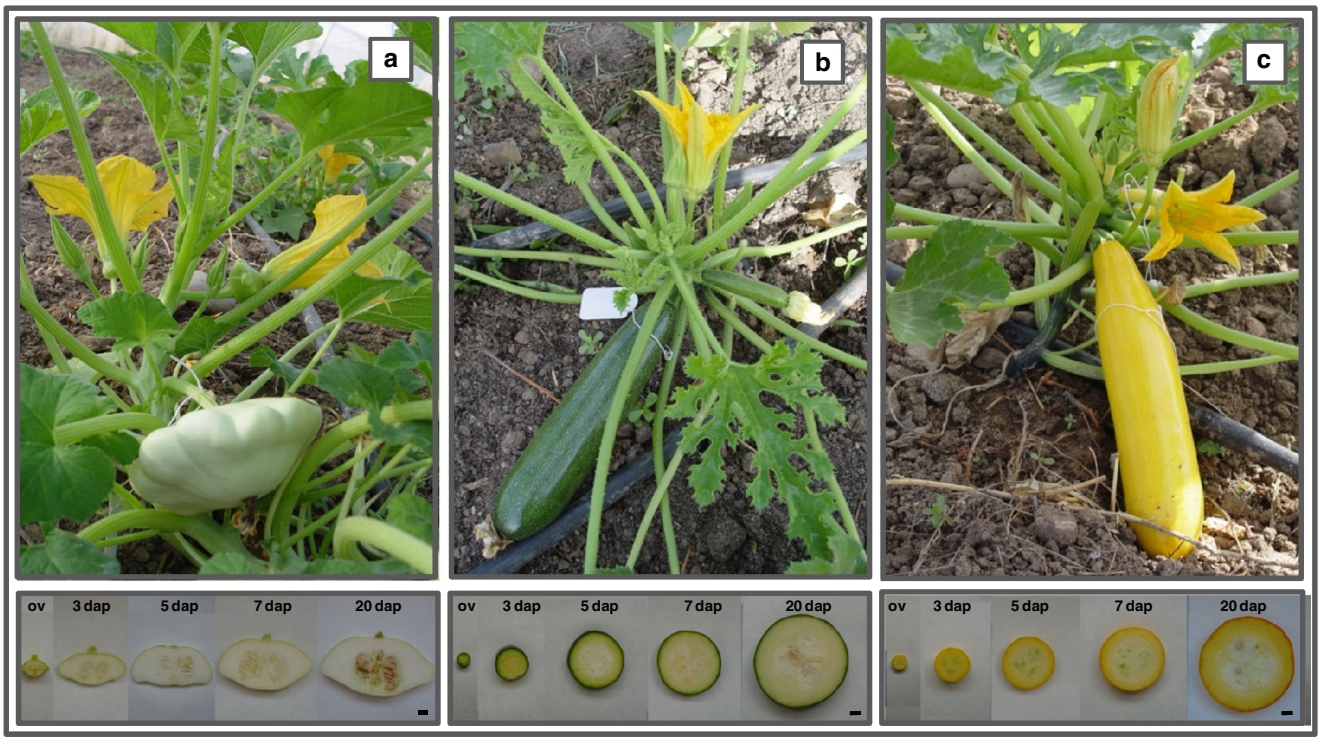

Fig. 1 Plant phenotypes as observed in the three varieties of summer squash used in this study: *C. pepo* ssp *ovifera* Scallop (**a**), *C. pepo* ssp *pepo* MU_CU16 (**b**) and *C. pepo* ssp *pepo* Parador (**c**). Cross section of the ovary (ov) and fruit at stages 3, 5, 7 and 20 days after pollination (dap) is detailed. *Scale bar* represents 1 cm

Only RNA samples with high quality presenting RQI>8 were used for cDNA synthesis. 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. A negative control containing all of the reaction components except the reverse transcriptase was included for each sample to detect possible genomic DNA contamination.

Analysis of Gene Expression

CpNCED1, *2* and *3* EST sequences (accession numbers GU380290, GU380291 and GU380292, respectively) were identified from the NCBI GenBank Database (www.ncbi.nlm.nih.gov), while the remaining sequences were identified from the transcriptome sequencing from different summer squash tissues [20] and available in the Cucurbit Genomics Database (www.icugi.org). The unigenes PU049716, PU036433, PU059552, PU056555 and PU087980 were selected and, according to its closest *Arabidopsis* members, they were designated as *CpNCED9*, *CpCCD1*, *CpCCD4a*, *CpCCD4b* and *CpCCD8*. These sequences were used to design specific primer pairs to amplify about 100 bp length fragments, at optimal melting temperature (Tm) at 60 °C and GC contents between 35 and 65 % (Table 1). Quantitative PCR (qPCR) using SYBR Green technology was carried out in a Mx3000P Real Time PCR System (Stratagene, CA, USA). The qPCR reactions, containing 300 nM of each gene-specific primer, 1.5 μl of cDNA sample (≈10 ng of input RNA), and 2× iTaq Fast SYBR Green Supermix (Bio-Rad, CA, USA) were run. The thermal cycling conditions were polymerase activation (95 °C for 3 min) and amplification cycles (95 ° C for 30 s, 60 °C for 30 s, 40×). Amplification of the specific transcript was confirmed by the appearance of a single peak in the melting curve analysis following completion of the amplification reaction. All runs contained negative controls with no cDNA template for screening possible contamination. Expression levels were calculated using a relative quantification model with PCR efficiency correction, multiple reference gene normalization and use of error propagation rules [21]. *PP2A* and *EF1A* genes were used as control since they were previously selected as reliable reference genes for summer squash qPCR analysis [22].

Table 1 Primers used in the summer squash *CCD* genes expression analysis

Gene symbol	Sequence of qPCR primers (5′-3′)	Accession number
CpNCED1	F: aaatggagatcggagcttcaga R: acgtgccatgaaaaccatagg	GU380290 (NCBI)
CpNCED2	F: cacgtcgtgataccagatcatca R: cgaaccgggaagttttgttc	GU380291 (NCBI)
CpNCED3	F: tctccggtgacctatgacaaaga R: caatcaggaacgtcgatccatt	GU380292 (NCBI)
CpNCED9	F: tatggagatgccgattcgatt R: cccacaccacaacctcatgtt	PU049716 (CuGenDB)
CpCCD1	F: tacctggcaccacttcagaaga R: gttttggcatccacgacattc	PU036433 (CuGenDB)
CpCCD4a	F: aagggaaaattccgacgaaac R: gactttgcgtccatgactacga	PU059552 (CuGenDB)
CpCCD4b	F: agacgacggatatgttgtctcgta R: agcttcacgacagcgataatctc	PU056555 (CuGenDB)
CpCCD8	F: aaatggaattgtgtagggttgga R: ccattcctctgccatgttcat	PU087980 (CuGenDB)

Results and Discussion

Interest for high nutritional vegetables has prompted us to study putative genes controlling carotenoid accumulation in fruit of summer squash. In a previous work, where the same varieties were analysed, it was found that the principal carotenoids accumulated were lutein and β-carotene [6]. Moreover, carotenoids were more abundant in the peel and were found at high levels in MU_CU16 and at moderate levels in Parador. This last variety also accumulated these compounds in the flesh, while MU_CU16 presented very low levels. Carotenoids were absent in the peel and flesh of Scallop. In addition to this, expression responses of carotenogenic genes such as *LCYe* were associated with pigment fruit accumulation [6]. Nevertheless, the presence of transcritps of carotenogenic genes in the white variety Scallop, did not explain the absence of carotenoid in this genotype, suggesting that the biosynthetic pathway is possibly not the only mechanism responsible for the carotenoid accumulation in summer squash. For this reason, in this work we studied transcriptomic changes occurring in summer squash carotenoid breakdown genes. The results obtained for *CCD* genes were then compared to that obtained for genes involved in the biosynthesis as well as with respect to their carotenoid content within the three contrasting varieties.

Expression Profile of Carotenoid Cleavage Dioxygenase Genes in Different Organs

Relative transcript levels of *CpNCED1*, *2*, *3, 9*, and *CpCCD1*, *4a, 4b*, *8* genes involved in carotenoid cleavage were measured by qPCR in the three summer squash contrasting varieties Scallop, MU_CU16 and Parador. Several organs and tissues (leaf, flower before and after anthesis, ovary, as well as peel and flesh fruit at different developmental stages) were considered.

All genes, except *CpNCED2*, were expressed in some of the organs tested in the plant of summer squash. Therefore, given the lack of amplification for this gene in all the samples and in order to discard the possibility of non-specificity of the primers, a PCR assay was conducted by using genomic DNA.

Only a single band with the expected size was detected when genomic DNA was used as template. This sequence showed 89 % identity with watermelon NCED1 [15].

Low levels of *CpNCED1*, *CpNCED3* and *CpCCD8* transcripts were found in the three varieties, although in general their presence was not constant in all samples (Fig. 2). *CpNCED1* showed a differential expression at organ level and was specifically expressed in flowers before anthesis and ovaries. This transcript reached an average among the three varieties of 167 and 109-fold difference when compared with that obtained for flowers at anthesis and for the fruit in development, respectively. This result is in agreement with that obtained for homologous genes in chromoplast-rich organs [12]. On the other hand, *CpNCED3* transcript was increased 22-fold in the fruit of MU_CU16 while an induction of 3–4 folds was detected in leaves, ovary and fruit of this variety for *CpCCD8* gene with respect to Scallop and Parador.

Among the genes that showed intermediate or high expression in some organ, we found *CpNCED9, CpCCD1, CpCCD4a* and *CpCCD4b*. During the anthesis of flowers an increased level of *CpCCD1* transcript in all varieties and of *CpNCED9* in Scallop was found. Although *CCD1* was constitutively expressed along flower development in other species [23], *CpCCD1* transcript exhibited between 8 and 15-fold difference during anthesis, depending on the variety, when compared with that obtained for the flowers before anthesis. The predominant presence of this transcript in the flowers suggests that, as occurs in other plant species, CpCCD1 could

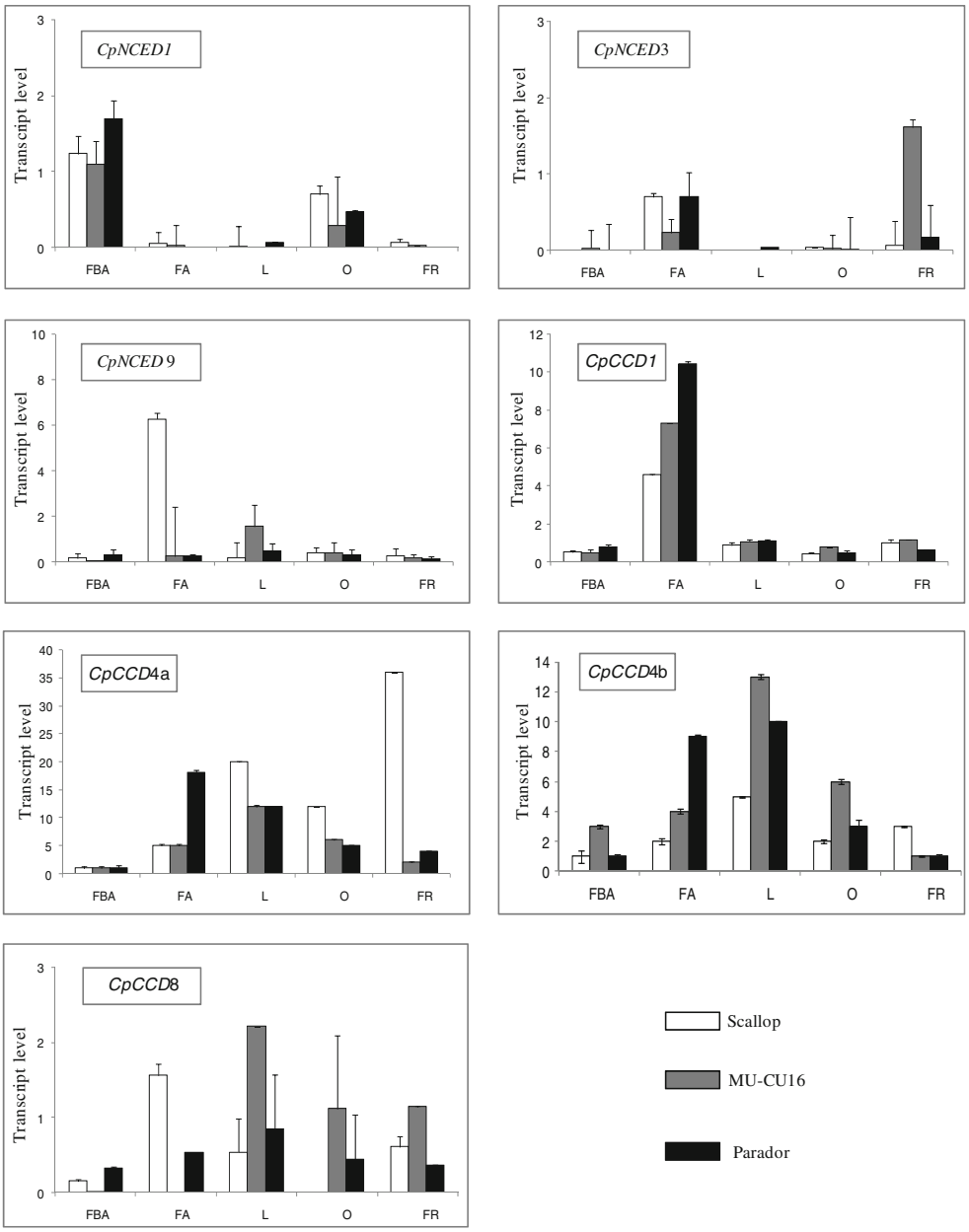

Fig. 2 Relative expression of carotenoid cleavage dioxigenase genes (*NCED1*, *NCED3*, *NCED9*, *CCD1*, *CCD4a*, *CCD4b* and *CCD8*) in different organs of the summer squash plant: flower before anthesis (FBA), flower in anthesis (FA), leaf (L), ovary (O) and fruit (FR). Fruit sample represents flesh at 5 day after pollination (M5), when this fruit is usually consumed. The three varieties with contrasting carotenoid content in flesh, Scallop, MU_CU16 and Parador were considered

be implicated in aroma production [24]. Finally, *CpCCD4a* and *CpCCD4b* were expressed in all organs and it was for *CpCCD4a* where an important up-regulation was observed in the fruit of Scallop that exhibited 17-fold difference with respect to the other varieties. *CpCCD4b* was also up-regulated in the fruit of Scallop but it was less prominent than that observed for *CpCCD4a*.

Carotenoid Cleavage Dioxygenase Gene Expression During Fruit Development

The analysis of transcript levels was conducted in four developmental stages of fruit from peel and flesh of Scallop, MU_CU16 and Parador. In general, these transcripts remained at low levels and only *CpNCED3*, *CpCCD4a, CpCCD4b, CpCCD1* and *CpCCD8* showed some variations among the fruit samples (Fig. 3).

CpCCD1 and *CpCCD8* transcripts were present in the three varieties but a direct relationship between their expression and carotenoid content was not found. *CpCCD8* showed little difference throughout the development. A slight transcript increase was observed in the peel of the three varieties, mainly in Parador. In the flesh, *CpCCD8* transcripts were also detected, and Scallop presented the higher level. In tomato it has been demonstrated that *SlCCD8* is involved in strigolactone biosynthesis and it is related to fruit development. Thus, reduced *SlCCD8* levels displayed smaller fruits with fewer seeds [25]. This might explain the expression of *CpCCD8* in the fruit peel and flesh, which could also be involved in the normal development of the fruit. *CpCCD1* transcript level was rather similar during all the stages of the fruit in the three varieties. Only an increase of more than 6-fold in the peel of Scallop and in the peel and flesh of Parador for the last stage of 20dap was observed. Since transcript accumulation showed

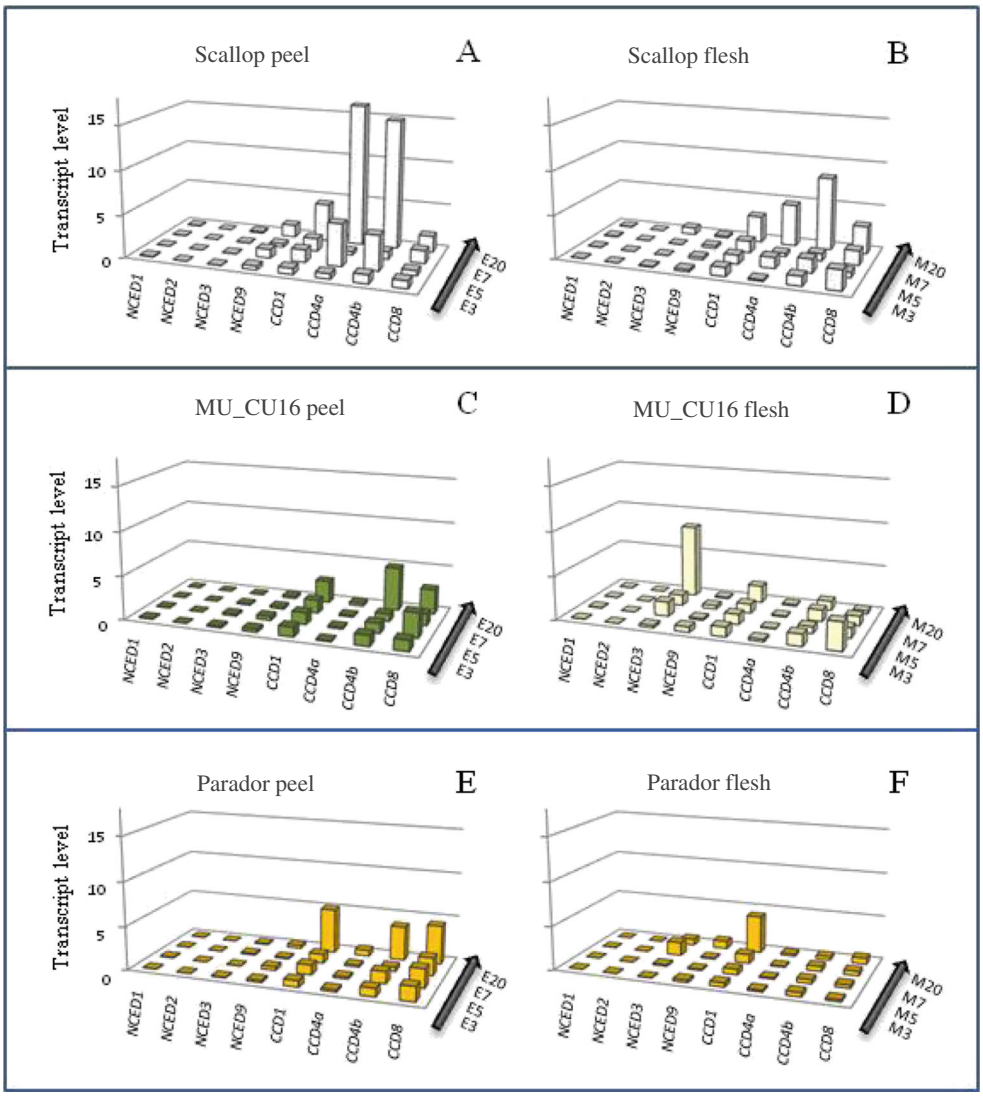

Fig. 3 Relative expression of genes involved in carotenoid degradation (*CpNCEDs and CpCCDs*: *NCED1, NCED2, NCED3, NCED9, CCD1, CCD4a, CCD4b* and *CCD8*) in summer squash fruit. Several development stages of the exocarp (E3-E20 in **a**, **c** and **e**) and mesocarp (M3-M20 in **b**, **d** and **f**) of the three varieties included in this study: Scallop (**a**–**b**), MU_CU16 (**c**–**d**) and Parador (**e**–**f**) are detailed

little difference among the three varieties, it seems unlikely that CpCCD1 could be implicated in carotenoid profile. This was also observed in other fruit [13] and it is possibly due to the fact that the activity of CCD1 proteins seems not to be located in the plastid like the other CCDs, but in the cytoplasm with less availability of substrate [26, 27].

Gene expression of *CpNCED3* was low both in the peel and flesh of the three varieties, except for the mature flesh of MU_CU16, where it was noteworthy that its transcript increased to 10-fold more than in the flesh of Scallop or Parador. This increase was found in the creamy-coloured flesh of this variety, but not in its green peel.

Only an apparent inverse relationship between gene expression and fruit carotenoid content was observed for *CpCCD4a* and *CpCCD4b*, suggesting that these genes could contribute in some way to the absence of carotenoids accumulation in the white tissues. *CpCCD4a* and *CpCCD4b* transcripts were expressed at a higher level in Scallop, where an inverse relationship between these CCD genes expression and fruit carotenoid content was observed. *CpCCD4a* transcript was highly abundant in this variety without the accumulation of carotenoids, reaching its maximum at the 20dap stage of 47-fold in peel and 31-fold in the flesh, more than that observed in the other varieties. *CpCCD4b* was also highly up-regulated mainly in the flesh of Scallop. Several studies have demonstrated that the loss of *CCD4* genes activity is related to an increased carotenoid content in *Chrysanthemum* flowers [28, 29] as well as in potato tubers and peach fruit [16–18]. It could be possible that these genes cleave synthesized carotenoids in this white flesh-peeled variety as occurs in other species.

Taking into account the results obtained in the preceding work it was observed that although most of the genes studied leading to carotenoids biosynthesis were expressed in the peel of the three varieties, a higher expression was found in the green peel of MU_CU16 and the yellow peel of Parador presenting higher carotenoid accumulation. The expression levels in the flesh of these varieties, compared with the peel, were lower and the colour variation was less evident [6]. Moreover, we found in this work that *CCDs* transcripts remained at relatively low levels in these tissues. These facts suggest that carotenoid biosynthesis might be regulating the accumulation of these pigments in these varieties. However, the process was different for the white variety Scallop where, although moderate levels of biosynthesis genes were found, an accumulation of *CpCCD4a* and *CpCCD4b* transcripts was observed. The reason why Scallop fruit tissues are unable to accumulate carotenoids, even though carotenogenic genes are expressed, could be that the cleavage of carotenoids by CCD4 enzymes may be more prominent than their biosynthesis.

Conclusions

The analysis of the *CCDs* expression patterns in the different organs suggested that these genes are differentially regulated and probably develop different functions in different tissues of summer squash. Thus, *CpCCD1* might regulate issues of flower development while *CpCCD4a* and *CpCCD4b* could be related with the presence of carotenoids in the peel and flesh of the fruit. Accumulation of carotenoids in the fruit of summer squash is the result of the combination of both regulation of biosynthesis and degradation as it has been seen in three varieties with high, medium and low carotenoid content [6]. *CpCCD4* gene members seem to be the most important in the regulation of the contents of carotenoids in the fruit but not the only one, which should be taken into account by breeders. Further studies of *CCD* genes and the development of molecular markers could help in breeding programs for the development of cultivars with higher content of desirable carotenoids.

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Conflict of Interest The authors declare no conflict of interest. This article does not contain any studies with human or animal subjects.

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