Involvement of ethylene in color changes and carotenoid biosynthesis in loquat fruit (*Eriobotrya japonica* Lindl. cv. Algerie)

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Short title: Ethylene, coloration and carotenoid metabolism in loquat fruit

Abstract

In loquat (*Eriobotrya japonica* Lindl cv. Algerie) fruit, despite the non-climacteric ripening behaviour, evidence suggest that ethylene may participate in the regulation of several ripening- and postharvest-related processes. Color changes and carotenoid profile were analyzed in fruit at three developmental stages (breaker, yellow and colored fruits). At early stages, the fruit peel contained phytoene, phytofluene and other typical chloroplastic carotenoids that decreased during ripening, to accumulate β-carotene, violaxanthin and β-cryptoxanthin in mature fruits. In the pulp, carotenoid concentration increased during ripening to become predominant phytoene, followed by β-carotene and β-cryptoxanthin. Expression of the carotenoid biosynthetic genes (*PSY*, *PDS*, *ZDS*, *CYCB* and *BCH*) was downregulated in the peel during maturation, but increased in the pulp with the exception of *BCH*. The involvement of ethylene in the regulation of pigmentation was further evaluated by treating fruits at the three ripening stages with ethylene or its action inhibitor 1-MCP. At breaker fruit, ethylene accelerated and 1-MCP delayed fruit coloration, but the effect was progressively lost as fruit matured. Ethylene and 1-MCP produced different changes in carotenoids content and gene expression in peel and pulp. Application of ethylene enhanced β-carotene content in both tissues whereas β-cryptoxanthin was only stimulated in the pulp. 1-MCP suppressed these changes in carotenoid composition in the pulp but had little effect in the peel. A differential transcriptional level the pulp was more responsive to downregulated gene expression than the peel. Collectively, results indicate that: 1) ethylene is involved in the regulation of pigmentation and carotenoid biosynthesis in loquat fruits, 2) a differential regulation of carotenoid biosynthesis and response to ethylene appear to operate in the peel and the pulp, and 3) β-carotene hydroxylase (*BCH*) is a key step in the regulation of carotenoid content and composition in both tissues of loquat fruit.

Keywords: carotenoids, ethylene, fruit, loquat, physiology, postharvest 1-MCP.

1. Introduction

Loquat (*Eriobotrya japonica* L.) belongs to the *Rosaceae* family, is native of Southern China and is currently cultivated in more than 20 countries, being China and Spain the main producers (Calabrese, 2006). Ripening of loquat fruits has been classified as non-climacteric with a virtual absence of a rise in the respiration rate and autocatalytic ethylene production (Jiang et al., 2011, Pech et al., 2012; Reig et al., 2016). Furthermore, the transcriptional regulation of ethylene biosynthetic and perception genes reinforces the notion of a non-climacteric ripening behavior (Alós et al., 2017). However, several studies in loquat have revealed the involvement of ethylene in some ripening-related events or during storage, such as accumulation of sugars, the reduction of acids, flesh browning, polygalactoronase (PG) activity, lignin accumulation and the appearance of chilling injury lesions (Cai et al., 2006; Wang et al., 2010, Liguori et al., 2015). In addition, experiments involving the application of ethylene or 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action, in loquat fruits revealed that ethylene seems to be required to sustain the expression of certain genes of its own biosynthesis (Alós et al., 2017).

External color is one of the main parameters of fruit quality and in loquat has been established as the main determinant for the harvest date (Cautín et al., 2006). Depending on the coloration of the flesh at mature stage, loquat fruits can be divided into two types: the white- and the red/orange-fleshed cultivars. The distinctive coloration of the fruit of the different varieties is due to the differential accumulation of specific carotenoids. The carotenoid biosynthetic pathway is well stablished in some fleshy fruits and the main metabolic steps have been characterized and extensively studied (Lado et al., 2016). Hence, carotenoids are formed from the 2-methyl-erythritol-phosphate (MEP) pathway which generates geranylgeranyl diphosphate (GGPP) that is then used to synthesize phytoene via phytoene synthase (PSY), the first committed step in carotenogenesis (Fig. 1). Subsequently, a series of desaturation and isomerization reactions catalyzed by phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), ζ-carotene isomerase (ZISO), and carotenoid isomerase (CRTISO), lead to the formation of

lycopene, the red-colored carotenoid. Lycopene β-cyclase (LCYB) and lycopene ε-cyclase (LCYE) together synthesize α-carotene or alternatively, a lycopene β-cyclase (LCYB) or a chromoplast-specific lycopene β-cyclase (CYCB) (Ronen et al., 2000; Alquézar et al., 2009) synthesize β-carotene (Fig. 1). The cyclization of lycopene to produce α-carotene or β-carotene leads to the bifurcation of the pathway into the α- and the β-branch, respectively. Then, the hydroxylation of α-carotene and β- carotene by β- and ε-carotene hydroxylases (BCH, CYP97A and B) generate yellow xanthophylls of lutein in the α-branch and zeaxanthin in the β-branch. The epoxidation and de-epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) constitute the so-called xanthophyll cycle. The conversion of violaxanthin into neoxanthin by neoxanthin synthase (NSY) concludes the core biosynthetic pathway (reviewed in Giuliano, 2017; Sun et al., 2017, Fig. 1).

The genetic and transcriptional regulation of the carotenoid biosynthetic pathway in fruits of loquat genotypes with different carotenoid accumulation patterns has been recently addressed. Studies on loquat cv. Luoyangqin (LYQ) and cv. Baisha (BS) with orange- and white-fleshed fruits, respectively, revealed significant differences in carotenoid content and composition in peel and pulp tissues between both cultivars associated with the differential expression of the carotenoid biosynthetic genes: *PSY1*, *CYCB* and *BCH* (Fu et al., 2012; Fu et al., 2014). Recently, Hadjipieri et al. (2017) analyzed the carotenoid composition and the expression of several carotenoid biosynthetic genes during on-tree maturation of the red/orange-fleshed cv. Obusa. During maturation, lutein content in the peel decreased progressively and increased the concentration of β-carotene, while β-cryptoxanthin and β-carotene were the main carotenoids in the pulp. Analysis of gene expression in peel and pulp of fruits at six developmental/maturation stages revealed that these changes in carotenoid content were linked to the coordinated upregulation of *CYCB* and the repression of *LCYB* and *LCYE* genes (Hadjipieri et al., 2017). Therefore, the shifting from the β,ε to β,β branch of the carotenoid biosynthesis pathway and the hydroxylation of β–carotene appear to be key steps in the regulation of carotenoid accumulation in loquat fruits.

Carotenoid synthesis and accumulation in fruits are influenced by different developmental and environmental factors (Lado et al., 2016). In climacteric fruits, ethylene plays a key role in the regulation of fruit coloration and carotenoid biosynthesis (Grierson, 2013). In non-climacteric fruits, however, the absence of an upsurge of ethylene production during maturation does not discard the involvement of the hormone in the regulation of fruit coloration. Indeed, in *Citrus* fruit, in which application of ethylene is commercial used worldwide to degreen early harvested cultivars (Porat, 2008), it has been suggested that ethylene action is required for the expression of carotenoids biosynthesis genes and peel coloration (Rodrigo and Zacarías, 2007; Rodrigo et al., 2013). Moreover, inhibition of ethylene perception by postharvest application of 1-methylcyclopropene (1-MCP), an inhibitor of the ethylene action, is a valuable experimental tool to clarify the role of endogenous ethylene on several ripening-related events, including fruit pigmentation (Watkins, 2008; Li et al., 2016). In particular, application of 1-MCP to loquat has been shown to reduce the incidence of postharvest disorders as chilling injury (internal browning and leatheriness) and decay (Pareek et al., 2013; Li et al., 2016) and also to delay ripening (Liguori et al., 2015). Together, these observations indicate that ethylene may be involved in specific aspects of the postharvest performance of loquat fruits. However, the involvement of the hormone in the regulation of fruit coloration and carotenoid biosynthetic in this fruit, and whether 1-MCP may be commercially used to manipulate fruit coloration, have not been elucidated. Hence, in the present study we have addressed two objectives: 1) to analyze carotenoid content and expression of key biosynthetic genes in peel and pulp of loquat (cv. Algerie) fruit during on-tree maturation, and 2) to investigate the effects of ethylene and 1-MCP during postharvest on color changes and carotenoid biosynthesis in both peel and pulp tissue of loquat fruits cv. Algerie, the predominant cultivar in Spain. Overall, the data suggest that, despite the non-climacteric ripening of loquat, coloration and carotenoid accumulation can be manipulated by modulation of ethylene action, most likely throughout transcriptional changes in carotenoid biosynthetic genes, but the effect appears to be tissue- and maturation stage-specific.

2. Materials and Methods

2.1. Plant material and treatments

Fruits were harvested from 6 adult (20-25 years old) trees of loquat cv. Algerie (*Eriobotrya japonica* Lindl.) grown in a commercial orchard in Callosa d'En Sarriá, (Alicante, Spain), the main area of loquat production in Spain. This cultivar was selected by the excellent quality of its fruit and because it is the one with highest production in the Mediterranean basin. Trees were budded onto loquat seedling rootstocks, grown in a loamy-clay soil, pH 7.5–8.0, planted 4 × 4 m apart, with drip irrigation (2 drippers per tree), and pruned to a vase shape. Fertilization, annual pruning, thinning, as well as pest and disease management, were carried out in accordance with standard commercial practices.

Fruits were sampled at breaker (BK), yellow (Y) and full color stages (FC), corresponding to the stages 801, 803, and 809 on the BBCH scale, respectively (Martínez-Calvo et al., 1999) or to the stages S3, S4 and S5, respectively, according Hadjipieri et al. (2017). Fruits were harvested and immediately delivered to the laboratory for tissue sampling. Twenty fruits were selected for uniformity and the absence of any lesion or injury. Peel and pulp color was determined on three locations around the equatorial plane of the fruit, using a Minolta CR-330 colorimeter. Color index was expressed as the *a/b* Hunter ratio (refered to as color index, Alos et al., 2017), which is negative for green fruit, around 0 for yellow fruit at color break and positive for orange-colored fruit. Peel and pulp were separated with a scalpel, immediately frozen in liquid nitrogen, ground to a fine powder and stored at -80ºC until RNA and pigment analysis.

Moreover, the effect of exogenous ethylene and of the ethylene action inhibitor, 1-MCP, on pigment accumulation and gene expression in peel and pulp was also evaluated in yellow-colored fruits (Y) at the growth stage 803 on the BBCH-scale. Fruits were divided into three lots, containing three replicate samples of 20 fruits each, and treated in air (control), ethylene or 1-MCP as previously described by Alos et al. (2017). After 0, 2 and 6 days of treatment, peel and pulp color of 20 fruits was determined and fruit tissue processed for RNA and pigment extractions as described above.

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159 *2.2. Chlorophyll and carotenoid extraction*

160 Peel (0.5 g fresh weight) and pulp (1.5 g fresh weight) fruit pigments were extracted as
161 previously described (Rodrigo et al., 2004; Carmona et al., 2012). The total chlorophyll (a+b)
162 content was determined by measuring the absorbance at 644 and 662 nm and calculated
163 according to the Smith and Benitez equations (Smith and Benitez, 1955). After chlorophyll
164 measurements, the pigment ethereal solution was dried and saponified using a 10% methanolic
165 KOH solution. Carotenoids were extracted and samples dried under N_2 and kept at -20º C until
166 analysis. All operations were carried out on ice under dim light to prevent photodegradation,
167 isomerisation and structural changes of carotenoids.

168

169 *2.3. Carotenoid analysis by HPLC*

170 Prior to HPLC analysis, carotenoid extracts were dissolved in acetone and incubated
171 overnight at -20º C to precipitate sterols which could interfere in the carotenoid analysis and
172 subsequently dried under N_2. The carotenoid composition of each sample was analyzed by
173 HPLC with a Waters liquid chromatography system equipped with a 600E pump and a model
174 996 photodiode array detector (PDA), and Empower software (Waters). A C30 carotenoid
175 column (250 × 4.6 mm, 5 µm) coupled to a C30 guard column (20 × 4.0 mm, 5 µm) (YMC
176 Europe GmbH) was used. Samples were prepared for HPLC by dissolving the dried carotenoid
177 extracts in $CHCl_3$: MeOH: acetone (3:2:1, v:v:v). A ternary gradient elution with MeOH, water
178 and methyl *tert*-butyl ether (MTBE) was used for carotenoid separation reported in previous
179 works (Carmona et al., 2012). Briefly, the initial solvent composition consisted of 90% MeOH,
180 5% water and 5% MTBE. The solvent composition changed in a linear fashion to 95% MeOH
181 and 5% MTBE at 12 min. During the next 8 min the solvent composition was changed to 86%
182 MeOH and 14% MTBE. After reaching this concentration the solvent was gradually changed to
183 75% MeOH and 25% MTBE at 30 min. Final composition was reached at 50 min and consisted
184 of 50% MeOH and 50% MTBE. Initial conditions were re-established in 2 min and re-
185 equilibrated for 15 min before next injection. The flow rate was 1 mL min^{-1}, column

186 temperature was set to 25 °C and the injection volume was 20 µl. The photodiode array detector
187 was set to scan from 250 to 540 nm, and for each elution a Maxplot chromatogram was
188 obtained, which plots each carotenoid peak at its corresponding maximum absorbance
189 wavelength. Carotenoids were identified by comparison of the spectra and retention time with
190 those of authentic standards, when available, or by matching the observed versus literature
191 spectral data and retention time under or similar identical chromatographic conditions (Rodrigo
192 et al., 2004). The carotenoid peaks were integrated at their individual maxima wavelength and
193 their content were calculated using calibration curves of β-cryptoxanthin (Extrasynthese), lutein
194 (Sigma), violaxanthin for violaxanthin isomers, luteoxanthin, zeaxanthin (Extrasynthese) and β-
195 carotene (Sigma) for β-carotene isomers. Standards of phytoene and phytofluene for
196 identification and quantification were obtained from extracts of sweet orange fruits cv. Pinalate,
197 which accumulate large amounts of these compounds (Lado et al., 2015), and neoxanthin
198 isolated from spinach extracts (Britton, 1995). Samples were extracted at least twice and each
199 analytical determination was replicated at least once. All operations were carried out on ice
200 under dim light to prevent photodegradation, isomerisations and structural changes of
201 carotenoids.
202
203 *2.4. RNA extraction and cDNA synthesis*
204 Total RNA was isolated from the fruit tissues using RNeasy Plant Mini Kit (Qiagen)
205 and subsequently treated with DNase I (DNA free, DNase treatment & removal, Ambion). The
206 amount of RNA was measured by spectrophotometric analysis (Nanodrop, Spain) and its quality
207 was verified by agarose gel electrophoresis and ethidium bromide staining. The absence of
208 DNA contamination was checked by performing a no-reverse transcription assay which
209 consisted of a PCR with each RNA sample using the *ACTIN* primers (Fu et al., 2012). No
210 amplified products were detected which confirmed the purity of the RNA extracts. The
211 transcripts present in 5 µg of total RNA were reverse-transcribed using the SuperScript III
212 Reverse Transcriptase (Invitrogen) in a total volume of 20 µL. One µL of a 10-fold diluted first-
213 strand cDNA was used for each amplification reaction.

214

215 *2.5. Gene expression analyses by real time PCR*

216 Gene expression analyses were performed following the MIQE guidelines (Bustin et al.
217 2009). Quantitative real-time PCR was carried out on a LightCycler 480 instrument (Roche),
218 using the LightCycler 480 SYBRGreen I Master kit (Roche). Reaction mix and conditions
219 followed the manufacturer's instructions with some modifications. The PCR mix contained 1 µL
220 of diluted cDNA, 5 µL of SYBR Green I Master Mix, 1 µL of 3 µM primer F and 1 µL of 3 µM
221 primer R, being the final volume of 10 µL. The sequences of the primers (PSF purified, Isogen,
222 The Netherlands) for the amplification of *PSY*, *PDS*; *ZDS*, *LCYB, CYCB*, *BCH* and *ACTIN* were
223 obtained from Fu et al. (2012). The cycling protocol, for all genes, consisted of 10 min at 95 °C
224 for pre-incubation, then 40 cycles of 10 sec at 95 °C for denaturation, 10 sec at 59 °C for
225 annealing and 10 sec at 72 °C for extension. Fluorescent intensity data was acquired during the
226 extension time with the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche) and
227 were transformed into mRNA levels by using specific standard curves for all analyzed genes.

228 The specificity of the PCR reaction was assessed by the presence of a single peak in the
229 dissociation curve performed after the amplification steps followed by the sequencing of the
230 amplicon. The expression levels relative to values of a reference sample were calculated using
231 the Relative Expression Software Tool (REST, http://rest.gene-quantification.info; Pfaffl et al.
232 2002). The reference sample was the expression value obtained for each gene on the pulp of the
233 loquat fruits at the yellow stage which was arbitrarily given the expression value of 1. Results
234 were the mean of at least 3 independent replicates.

235

236

237 **3. Results**

238 *3.1. Evolution of color index and pigment concentrations during on-tree loquat fruit maturation*

239 Color index (CI) data showed that fruit peel was greener than the pulp at BK stage (-
240 0.33 and -0.13, respectively, Fig. 2A and B). As fruit maturation progressed, external
241 pigmentation and CI increased, being higher in the peel than in the pulp (Fig. 2A and B). Total

carotenoid and chlorophyll content was also measured at these maturation stages and indicated a higher pigment concentration in the peel than in the pulp. Total carotenoids in the peel were almost the same in Y and BK fruits, and decreased at FC stage, while in the pulp carotenoid concentration increased from 1 to 9 mg kg^{-1}, from BK to FC fruits, respectively (Fig. 2C). The concentration of total chlorophylls in the peel decreased dramatically from the BK to the Y stage (51 to 18 mg kg^{-1}, respectively) while in the pulp at theses two stages was around 1 mg kg^{-1}. Chlorophyll was not detected in peel and pulp of full-colured fruits (Fig. 2D).

Twenty-four different carotenoid-like peaks were detected by HPLC coupled to a PDA in the peel and pulp extracts of 'Algerie' loquat fruit (Table 1). Ten carotenoids, 15-*Z*-phytoene, phytofluene, all-*E*-neoxanthin, all-*E*- and 9-*Z*-violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene, were unambiguously identified by comparing chromatographic and spectroscopic characteristics with those of authentic standards. Luteoxanthin, a putative artifact derived from violaxanthin, several -isomers of β-carotene and phytoene-like were tentatively identified. The remaining carotenoid-like compounds showed the characteristic carotenoid absorption spectrum but were not ascribed to a specific carotenoid. The main carotenoid in the peel of BK and Y fruit was 15-*Z*-phytoene with concentrations close to 40 mg kg^{-1} followed by β-carotene (between 8 and 12 mg kg^{-1}) and lutein (between 9 and 6 mg kg^{-1}), whereas in FC fruit it was β-carotene followed by lutein and 9-*Z*-violaxanthin (Fig. 3). In the peel of fully ripe fruit, the concentration of 15-*Z*-phytoene was markedly lower (6 mg kg^{-1}), being β-carotene and its *Z*-isomers the predominant carotenoids, reaching values close to 25 and 6 mg kg^{-1}, respectively. Other xanthophylls like lutein or violaxanthin (9*Z* and all-*E* isomers) were also abundant (5 mg kg^{-1}) in the peel of FC fruit (Fig. 3). The concentration of all individual carotenoids, except that of β-cryptoxanthin, was lower in the pulp than in the peel at all fruit stages and, in addition, the pattern of accumulation of the carotenes 15-*Z*-phytoene and phytofluene was opposite between both tissues. Hence, whereas 15-*Z*-phytoene and phytofluene decreased in the peel during maturation, they increased in the pulp (Fig. 3). The concentrations of carotenoids with provitamin A activity, as β-carotene, their isomers, and β-cryptoxanthin

were enhanced during maturation of both tissues, whereas violaxanthin (all isomers detected) were relatively stable in the peel and increased in the pulp. It is worth to note that lutein was one of the most abundant carotenoids in the peel while it was barely detectable in the pulp at any stage analyzed (Fig. 3). Zeaxanthin was only detected at trace levels (data not shown). At FC stage, the predominant carotenoid in the pulp was 15-*Z*-phytoene (3 mg kg^{-1}) followed by β-carotene and β-cryptoxanthin, with concentrations around 1.5 mg kg^{-1} (Fig. 3).

3.2. *Expression of genes involved in carotenoid biosynthesis during on-tree loquat fruit maturation*

The expression profile of six key genes of the carotenoid biosynthetic pathway in the peel and pulp of 'Algerie' loquat fruit at the three maturation stages was measured by RT-qPCR. These genes included the first committed step of the carotenoid pathway *PSY,* the two subsequent desaturation activities prior to cyclization, *PDS* and *ZDS;* the chromoplast specific *lycopene β-cyclase* (*CYCB*)*,* and *β-carotene hydroxylase* (*BCH*). The expression the *lycopene β-cyclase* (*LCYB*) gene, which participates together with *LCYE* in the formation of *β*- and α-carotene (Fig 1), was also analyzed but their expression levels were negligible in both tissues throughout the whole process studied (data not shown).

The relative expression levels of *PSY*, *PDS*, *ZDS* and *CYCB* were higher in the peel than in the pulp at BK and Y stages, but not at FC stage, for which the transcript levels of all genes analyzed was higher in the pulp. It is interesting to note that the pattern of expression of these genes was different for the two tissues, peel and pulp (Fig. 4). In general, transcript accumulation in the peel decreased during maturation, whereas abundance of the *PSY*, *PDS*, *ZDS* and *CYCB* transcripts significantly increased in the pulp at BK stage, declined at Y stage and increased again at FC stage. Expression of the *BCH* gene was the exception, since it was lower in the peel, and its maximum level in the pulp was attained at the BK stage, decreasing gradually during fruit maturation (Fig. 4).

296 *3.3. Effect of ethylene and 1-MCP on color and carotenoid content in peel and pulp of loquat*
297 *fruit during postharvest storage*

298 In order to gain insights on the role of ethylene in the regulation of carotenoid
299 biosynthesis and accumulation in loquat, fruits at three maturation stages were incubated during
300 postharvest in an ethylene atmosphere (10 μl L^{-1}) up to 6 days at 20 °C and 85–90% RH in the
301 dark, or treated with the inhibitor of ethylene action 1-MCP (1 μl L^{-1}) for 16 h, and then
302 exposed to air in the dark.

303 The effects of exogenous ethylene and 1-MCP were firstly evaluated by measuring
304 changes in peel and the pulp color of fruits 2 and 6 days after the exposure to these treatments
305 (Fig. 5). In the peel of fruits at the BK stage (initial a/b ratio of -0.34) degreening was well
306 patent in non-treated fruit, and accelerated by ethylene (Fig. 5A). However, 1-MCP
307 considerably delayed fruit coloration and 6 days after treatment fruit became greener than non-
308 treated fruit (Fig. 5A). The pulp of these fruits displayed similar responses to ethylene and 1-
309 MCP, accelerating and delaying, respectively, degreening with respect to untreated fruits (Fig.
310 5A). In Y fruits, the responses to ethylene and 1-MCP were less pronunciated that in BK fruits,
311 and interestingly similar in peel and pulp (Fig. 5B). In colored fruits (FC stage), ethylene and 1-
312 MCP did not induce significant differences compared to untreated fruits (Fig. 5C). Collectively,
313 these results indicated that ethylene action appears to be involved in color changes of loquat
314 fruit at the onset of fruit coloration, and as fruit matures the effect is progressively lost.

315 In order to understand the mechanisms of ethylene in the regulation of carotenoids
316 biosynthesis and accumulation, carotenoid content and gene expression were studied in peel and
317 pulp of Y fruit, because they retained significant effects of ethylene and 1-MCP and also it is
318 the stage of commercial harvest in loquat. Total carotenoids content was 15-fold higher in the
319 peel than in the pulp, with phytoene, β-carotene and lutein predominating in the peel, and
320 phytoene, β-carotene and β-cryptoxanthin in the pulp (Fig. 6A and B). Two and 6 days after
321 incubation in air, carotenoid content in the peel declined by about 60%, mainly by the reduction
322 of phytoene, but the composition was almost the same. Ethylene doubled the content of β-
323 carotene (all-E and *Z*-isomers), compared with freshly harvested or air-treated fruits, while

concentration of other carotenoids did not change significantly (Fig. 6A). The treatment with 1-MCP almost did not affect the composition of carotenoids in the peel respect to untreated (air) fruits (Fig. 6A).

In the pulp of freshly harvested fruits at Y stage, phytoene accounted for about 48% of the total carotenoids, and after 2 and 6 days in air, a 10- and 20-fold reduction, respectively, was registered (Fig. 6B). These changes were paralleled by an increase of β-carotene, β-cryptoxanthin and 9-*Z*-violaxanthin (Fig. 6). Ethylene increased around 40% the concentration of β-carotene and β-cryptoxanthin concentration at day 6, whereas the inhibitor of ethylene action markedly reduced the content of carotenoids in the pulp at day 2, specially β-carotene, β-cryptoxanthin and violaxanthin. At day 6 the, total carotenoids content increased again and the composition was similar to that of untreated fruits, although with lower values (Fig. 6B).

3.4. Effect of ethylene and 1-MCP on the expression of carotenoid biosynthetic genes in peel and pulp of loquat fruit

The expression of carotenoid biosynthetic genes in peel and pulp of control, ethylene- and 1-MCP-treated in Y fruit were analyzed. The transcript levels of *PSY*, *PDS*, *ZDS* and *CYCB* were lower in the pulp than in the peel in all the dates analyzed. In contrast, *BCH* transcription level was higher in the pulp (Fig. 7). As in previous experiments, *LCYB* transcripts were not detected in any sample analyzed.

In the peel of air-treated fruits, *PSY* transcript experienced a 1.75-fold increase at day 6 (Fig. 7). Ethylene provoked a 2-fold induction at day 2 compared to freshly harvested fruit, that was sustained at day 6. By contrast, 1-MCP initially repressed *PSY*, that nevertheless was restored at day 6. The expression of *PDS* was slightly increased at day 2 in the peel of ethylene and 1-MCP-treated fruits, while no important changes were observed in air-treated fruit (Fig. 7). *ZDS* and *CYCB* presented similar expression profiles, increasing in air and enhanced by ethylene, whereas 1-MCP diminished it (Fig. 7). The expression of *BCH* gene in the peel was temporally (day 2) reduced in untreated (air) and in 1-MCP-treated fruits, while no important changes were detected in ethylene-treated fruits (Fig. 7).

In the pulp, accumulation of *PSY* transcript was virtually unaffected in air- and ethylene-treated fruits, while 1-MCP produced a marked repression (Fig. 7). *PDS* was also constitutively expressed in air, and repressed in both ethylene and 1-MCP treatments at the end of the experiment (Fig. 7). *ZDS*, *CYCB* and *BCH* gene expression was increased by air and ethylene compared to freshly harvested fruits, while they were practically unaffected by 1-MCP (Fig. 7).

5. Discussion

In loquat, changes in coloration, i.e. in carotenoid content and composition in peel and pulp, during fruit maturation, are mainly due to transcriptional regulation of carotenoid biosynthetic genes (De Faria et al., 2009; Fu et al., 2014; Hadjipieri et al., 2017). Despite recent evidence that indicate a non-climacteric ripening behavior of loquat fruit, other results also suggest that several ripening-related processes could be potentially manipulated by either exogenous ethylene or by inhibitors of its action (Cai et al., 2006; Li et al., 2016; Alós et al., 2017). Thus, treatment of loquat fruits with 1-MCP has been reported to reduce the incidence of a number of physiological disorders and to maintain other parameters of internal fruit quality (Pareek et al., 2013; Liguori et al., 2015; Li et al., 2016), suggesting that these processes may be, at least under postharvest storage, partially regulated by ethylene. However, the effects of either 1-MCP or ethylene on color changes and carotenoid composition and biosynthesis in loquat is not fully understood. Our rationale to address these objectives was, first, to study changes in carotenoid content and in the expression of key biosynthetic genes during natural maturation at three stages (breaker, yellow and light colored-fruit), and second, to analyze the effect of ethylene (10 μl L^{-1}) and 1-MCP (1 μl L^{-1}) on these parameters in peel and pulp of loquat fruit harvested at the yellow stage.

Total carotenoids were similar in the peel of BK and Y fruits, but the presence of chlorophylls in the former masked the orange coloration associated with carotenoids (Fig. 2). Moreover, at both maturation stages, phytoene, a colorless carotene, was more than 50% of total

carotenoids that may explain the lower coloration in the peel than in the pulp (Fig. 2 and 3). In more mature fruits (FC), color was similar in pulp and peel (a/b ratio close to 0.2) although total carotenoids were 5-times lower in the pulp (Fig. 2). It is interesting to note that at this stage, β-carotene, an orange colored carotene, accounted for nearly 50% of the total content in the peel, while in the pulp orange-colored carotenoids (β-carotene plus β-cryptoxanthin) were about 35%, and phytoene and phytofluene accounted for the additional 40% (Fig. 3). These data highlight the fact that color measurements (usually as a/b Hunter parameters) in peel and pulp of loquat fruits are not well related to carotenoid concentrations, being more likely related to the complement in specific carotenoids. In fruit of other loquat cultivars, as 'Obusa', it has been also found that peel and pulp color of overripe fruits was identical while total carotenoid concentration was 6-times higher in the peel (Hadjipieri et al., 2017).

During on-tree maturation, striking differences in total carotenoid content were detected between loquat fruit tissues, being more abundant in the peel than in the pulp (from 5- up to 82-fold, Fig. 2C), similarly to that observed in other loquat cultivars ('Obusa', 'LYQ' and 'BS') (Fu et al., 2012; 2014; Hadjiperi et al., 2017) or other carotenogenic fruits, such as tomato or citrus (Carrillo-López and Yahia, 2014; Lado et al., 2016). These observations are in agreement with the higher expression levels of most carotenoid biosynthetic genes in the peel than in the pulp (Figs. 2 and 4).

Total carotenoid content varies greatly among cultivars, deepening on the red/orange coloration of the flesh. For example, the peel of ripe fruits of cv. 'Algerie' contains intermediate amount of total carotenoids (50 mg kg^{-1}), compared with those of 'LYQ' (70 mg kg^{-1}) and 'Obusa' (24 mg kg^{-1}). By contrast, differences in total carotenoid contents in the pulp among varieties are less pronounced, ranging from 9 mg kg^{-1} in 'Algerie' (Fig. 2), 12-13 mg kg^{-1} in 'Obusa' and 'LYQ' (Hadjipieri et al., 2017), 15 mg kg^{-1} in 'Centenaria' (Fu et al., 2012) to 20 and 30 mg kg^{-1} in Brazilian cultivars ('Mizauto', 'Mizuho' and 'Mizumo') (De Faria et al., 2009).

The concentration of individual carotenes and xanthopllys in peel and pulp of 'Algerie' loquat fruit followed different patterns during maturation. The evolution in the peel was similar

to a transition from a chloroplastic to a chromoplastic tissue, with increases in β-carotene (including its *Z*-isomers) and β-cryptoxanthin and reduction in lutein, like in other cultivars (Fu et al., 2014; Hadjipieri et al., 2017). It is noteworthy the elevated concentration of phytoene in the peel at BK and Y stages and the sharp reduction at FC stage (Fig. 3). In the pulp, by contrast, the concentration of phytoene increased during maturation, becoming the main carotenoid in mature fruits. In general, little attention has been previously paid to colorless carotenes although are commonly present in many fruits, and increasing evidences suggest they provide health-related benefits (Meléndez-Martínez et al., 2015). In fruits of other loquat cultivars, the presence of colorless carotenes has been described but data are fragmentary and only at specific maturation stages (De Faria et al., 2009; Fu et al., 2014; Hadjipieri et al., 2017). Thus, it will be interesting to consider these carotenes when carotenoid composition in loquat samples are analyzed to better understand how their biosynthesis and accumulation is regulated.

The increase in β,β-carotenoids and the reduction of the linear phytoene and phytofluene in the peel during maturation suggest that colorless carotenes are metabolized into downstream carotenoids. Consistent with this is the progressive decrease in *PSY,* the first committed step of the pathway, and the maintenance or slight reduction of *CYCB* and *BCH* levels during maturation, that may explain the decrease in phytoene and the accumulation β-carotene and β-cryptoxanthin (Figs. 3 and 4). By contrast, all the individual carotenoids increased in the pulp concomitantly with maturation, except luteoxanthin. The expression profile of *PSY*, *PDS*, *ZDS* and *CYCB* in the pulp was rather stable in BK and Y stages, and increased significantly at FC, when the highest carotenoids concentration was reached (Figs. 3 and 4). The expression of lycopene β-cyclase (*LCYB*) was not detected in the samples analyzed, in agreement with the results reported for fruits of other cultivars at similar maturation stage (Fu et al., 2012, Hadjipieri et al., 2017). Interestingly, in the pulp of cv. 'Algerie' the expression of *BCH* decreased to a very low level in mature fruits, showing an inverse relationship with the accumulation of the direct substrate, i.e. β-carotene (Figs. 3 and 4). These results reinforce the

key role of this step regulating carotenoid composition in the pulp of loquat fruit (Fu et al., 2014; Hadjipieri et al., 2017).

Accumulation of β-cryptoxanthin, a carotenoid with provitamin A and high bioavailability, is limited to a few number of fruits, as mandarins, sweet oranges or papaya (Lado et al., 2016). The xanthophyll β-cryptoxanthin is an intermediate product in a two-step hydroxylation by *BCH*, and has been proposed to accumulate predominantly under reduced or insufficient BCH activity, favouring monohydroxilation of β-carotene (Sun et al., 1996; Ikoma et al., 2016; Lado et al. 2016). Our results suggest that the accumulation of β-cryptoxanthin in loquat appears to be also governed by a similar mechanism, in which the downregulation of *BCH* expression as fruit matures would reduce hydroxilase activity and enhance accumulation of the intermediate product β-cryptoxanthin. Taking together, a differential regulation of carotenoid biosynthesis appears to operate in peel and pulp of loquat fruit, and *BCH* is likely a limiting step determining carotenoid composition in the pulp.

The involvement of ethylene in the regulation of loquat fruit coloration was firstly assessed by analyzing its effect and that of its antagonist 1-MCP on color changes in the peel and pulp at three maturation stages (Fig. 5). While ethylene accelerated fruit coloration, particularly at BK and being less effective at Y stage, 1-MCP delayed it regarding to the air-treated fruit, but a negligible effect was found in colored fruit. These results suggest that ethylene appears to be involved in the regulation of loquat fruit coloration and the BK stage (breaker) develops the optimum response, which is progressively lost as natural maturation progress. This situation resembles that referred to as ‘competence to ripen’ in climacteric-type fruit in which maximum sensitivity to ethylene-induced ripening changes is attained at a specific developmental stage, as breaker in tomato fruit (Klee and Giovannoni, 2011). Since loquat fruit is commercially harvested at a peel color around breaker to yellow, commercial treatment with 1-MCP to modulate color development and marketability may be feasible.

The effect of ethylene and 1-MCP on carotenoid content and composition, and on the expression of the biosynthesis genes in detached fruit, revealed differential regulation of the

461 pathway and responses to the hormone between peel and pulp (Figs. 6 and 7). At harvest,
462 phytoene represented around 60% of total carotenoids content in both tissues, and only after 2
463 days of storage in air it disappeared or was severely reduced in the peel and pulp, respectively
464 (Fig. 6). This reduction of phytoene content paralleled an increase of that of β-carotene and β-
465 cryptoxanthin in the pulp, but not in the peel that remained almost unaltered. Interestingly, in
466 ethylene-treated fruit, these changes in carotenoid composition were magnified with large
467 increments of β-carotene in both tissue (with the exception of pulp at day 2) and in β-
468 cryptoxanthin in the pulp and, in a lower extent, in the peel. The pulp seems to be more
469 sensitive to the 1-MCP-induced changes in carotenoid content and composition than the peel.
470 These changes suggest that, 1) differences in the decrease of phytoene content in the peel and
471 pulp appears to be an ethylene-independent event provoked probably by fruit harvest, 2) the
472 accumulation of β-carotene and β-cryptoxanthin is stimulated by ethylene in both tissues, and 3)
473 ethylene-dependent factors seems to operate in the accumulation of these carotenoids during
474 natural coloration of the pulp. Taken together, these observations reinforce the hypothesis that
475 ethylene is, at least in part, involved in the regulation of carotenoid accumulation in loquat, with
476 a differential response in peel and pulp tissues.
477 With the exception of *BCH*, most of the carotenoid biosynthetic genes evaluated in the
478 current study were more highly expressed and more responsive to treatments in the peel than in
479 the pulp. Nevertheless, the gene expression trends induced by ethylene and 1-MCP were, in
480 general, similar in both tissues (Fig. 7), and in agreement with the changes promoted by both
481 treatments in carotenoid content and composition. After fruit harvest and incubation in air, *PSY*,
482 *ZDS* and *CYCB* were upregulated in the peel. In the pulp, *ZDS*, *CYCB* expression was slightly
483 increased, whereas that of *BCH* experienced a major increment (about a 3-fold increment with
484 respect to the pulp of freshly-harvested fruit) (Fig. 7). Ethylene accelerated these changes and,
485 in general, the effects were higher in the peel than in the pulp. In other non-climacteric fruits,
486 such as *Citrus*, it has been also reported that *lycopene β-cyclase* was upregulated by postharvest
487 ethylene treatments (Rodrigo et al., 2007; Matsumoto et al. 2009). Therefore, the rapid and

488 substantial induction of *PSY*, especially in the peel, suggests the conversion of the linear
489 carotene phytoene into subsequent downstream products of the pathway, and explains its
490 decline in the peel of detached fruit. Other downstream genes of this pathway were also
491 upregulated, but *BCH* suffered only minor changes and, then, it is likely that the enhanced
492 metabolites flow would not be efficiently metabolized and, therefore, the substrate of this step,
493 β-carotene, accumulated in the peel. Since ethylene enhanced the expression of *PSY*, *ZDS* and
494 *CYCB*, without virtual effect on *BCH*, β-carotene may accumulate to a lager extend than in air-
495 treated fruits. The reduced effect of 1-MCP on the expression of these genes may justify that the
496 content and complement of carotenoids were similar to control fruit.

497 Comparison of the expression of carotenoids biosynthetic genes between peel and pulp
498 revealed that, whereas most of the transcripts were more highly expressed in the peel than in the
499 pulp, only *BCH* transcript accumulated to higher levels in the pulp (Fig. 7). This remarkable
500 difference between both loquat fruit tissues may be related to the differential carotenoid
501 complement. It is reasonable to assume that the moderated increase in the expression of genes
502 upstream *BCH* (*PSY*, *ZDS* and *CYCB*) would challenge the metabolic flow through the pathway,
503 increasing the concentration of β-carotene, and the important stimulation of BCH activity would
504 favor an efficiently conversion of β-carotene into β-cryptoxanthin. This scenario may explain
505 the increase in β-cryptoxanthin only in pulp of loquat fruit. Increasing expression of *BCH* has
506 been also associated with the accumulation of β-cryptoxanthin in persimmon (Zhou et al.,
507 2011). These results reinforce the concept that *BCH* is a key step in the regulation of carotenoid
508 content and composition in both peel and pulp of loquat. However, the involvement of other
509 genes and post-transcriptional modifications that are still unknown in the regulation of
510 carotenoid biosynthesis should not be discarded, as it has been demonstrated in *Arabidopsis*,
511 and tomato and other fleshy fruits (Fu et al., 2012; Zhou et al., 2015; Chan-León et al., 2017;
512 Rodriguez-Concepción et al., 2018).

513 Finally, application of 1-MCP also revealed a differential regulation of carotenoid
514 biosynthetic genes by endogenous ethylene between peel and pulp. In the pulp *PSY*, *ZDS*, *CYCB*

and *BCH* transcript levels were decreased significantly with respect to air-treated fruits, whereas in the peel only *ZDS* and *BCH* displayed a consistent reduction by 1-MCP (Fig. 7). These results suggests that endogenous ethylene is mediating the regulation of carotenoid biosynthesis in loquat fruit, but responsiveness to the hormone is different in both tissues. This situation appears not to be specific for carotenoid biosynthesis since in a previous report it was shown that inhibition of ethylene perception severely affected ethylene biosynthetic genes expression in the pulp of loquat while in peel the effect was less significant (Alós et al., 2017). Therefore, it can be speculated that in loquat fruit the pulp may be more sensitive to ethylene than peel tissue. The pulp of loquat also responds markedly to 1-MCP in the modulation of other postharvest processes, such as reduction of flesh browning and storage-induced loss of firmness (Cai et al., 2006, Liguori et al., 2015). It would be interesting to further investigate the role of ethylene in the development chilling injury in loquat upon storage at low temperatures.

7. Acknowledgements

Dr. E. Alos was recipient a post-doctoral contract JAE-Doc-CSIC (Fondo Social Europeo). The financial support of the research grants FP7-PEOPLE-2011-CIG-2011-303652 (Marie Curie Actions, European Union), AGL-2015-70218 (Ministerio Economía y Competitividad, Spain), GV/2012/036 (GeneralitatValenciana, Spain) and PROMETEO-II 2014/27 (Generalitat Valenciana) are gratefully acknowledged. MJR and LZ are members of Eurocaroten (COST_Action CA15136) and CaRed (Spanish Carotenoid Network, BIO2015-71703-REDT and BIO2017-90877-REDT).

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669 **Table 1.** Spectroscopic characteristics of the main carotenoids identified in the peel and pulp of
670 loquat fruit 'Algerie' during ripening. The letter `s´ indicates that there is a shoulder in the
671 spectrum. Bold letters indicate the carotenoids identified by using a standard, and carotenoids
672 with asterisk are tentatively identified.
673

		Observed	**Reference**
	Carotenoid	**λ_{max} (nm)**	**λ_{max} (nm)**
1	**All-E-Neoxanthin**	417,439,469	412,434,464
2	Not identified	400,421,445	
3	**All-E-Violaxanthin**	412,438,469	414,442,472
4	Not identified	S,432,459	
6	Not identified	401,422,447	
5	Not identified	413,435,464	
7	Luteoxanthin*	397,420,448	400,422,450
8	Not identified	405,439,467	
9	Not identified	398,401,425	
10	Phytoene-like*	285	
11	**9-Z-Violaxanthin**	(Z)325,415,435, 464	(Z)326,416,440,465
12	Not identified	398,418,443	
13	Not identified	402,438,469	
14	**Lutein**	420,444,472	421,445,474
15	**Zeaxanthin**	427,450,477	428,450,478
16	**15-Z-Phytoene**	285	276,286,297
17	Phytoene isomer*	285	
18	**Phytofluene**	330,347,364	331,348,367
19	**All-E-βCryptoxanthin**	427,450,477	428,450,478
20	Not identified	(Z)338,s,450,s	
21	13-Z-βCarotene*	(Z)337,s,444,470	(Z)338,s,444,470
22	**α–Carotene**	420,445,470	422,450,473
23	**All-E-βCarotene**	s,452,478	s,450,477
24	9-Z-βCarotene*	(Z)340,s,447,473	(Z)339,445,473

674

675 **Figure legends**

676 **Figure 1.** Overview of the carotenoid biosynthetic pathway in plants. Some steps are omitted
677 for simplification. The gene expression of the underlined enzymes has been measured by real
678 time PCR. Geranylgeranyl diphosphate, GGPP; phytoene synthase, PSY; phytoene desaturase,
679 PDS; ζ-carotene desaturase, ZDS; lycopene β-cyclase, LCYB; chromoplast specific lycopene β-
680 cyclase, CYCB; lycopene ε-cyclase, LCYE; β-carotene hydroxylase, BCH; β-carotene
681 hydroxylase; ε-carotene hydroxylase, ECH; violaxanthin de-epoxidase, VDE; zeaxanthin
682 epoxidase, ZEP; and neoxanthin synthase, NSY.

683

684 **Figure 2.** External and internal appearance (A), color index (expressed as Hunter *a/b*, B), total
685 carotenoids (mg kg^{-1}, C) and chlorophylls (mg kg^{-1}, D) of loquat fruit cv. Algerie (*Eriobotrya*
686 *japonica* Lindl.) at breaker, yellow, and full color stages (BK, Y, FC). These ripening stages
687 correspond to the stage 801, 803 and 809 of the BBCH-scale. Black and grey bars represent the
688 data for the peel and the pulp, respectively. Color index data are the mean ± S.E. of 20 fruits.
689 The pigment concentrations are means ± S.E of at least 3 replicates.

690

691 **Figure 3.** Carotenoid concentration in peel (black bars) and pulp (grey bars) of loquat fruit cv.
692 Algerie (*Eriobotrya japonica* Lindl.) at breaker (BK), yellow (Y) and full color stages (FC)
693 expressed as mg kg^{-1}. The data are means ± S.E of at least 3 replicates. For clarification
694 purposes the plots were arranged following the carotenoid biosynthetic sequence in the pathway
695 and geometric isomers for the same carotenoid are located at the same level. The 13-*Z*-β-
696 carotene and 9-*Z*-β-carotene have been added and presented as *Z*-isomers of β-carotene Tr,
697 traces; nd, not detected..

698

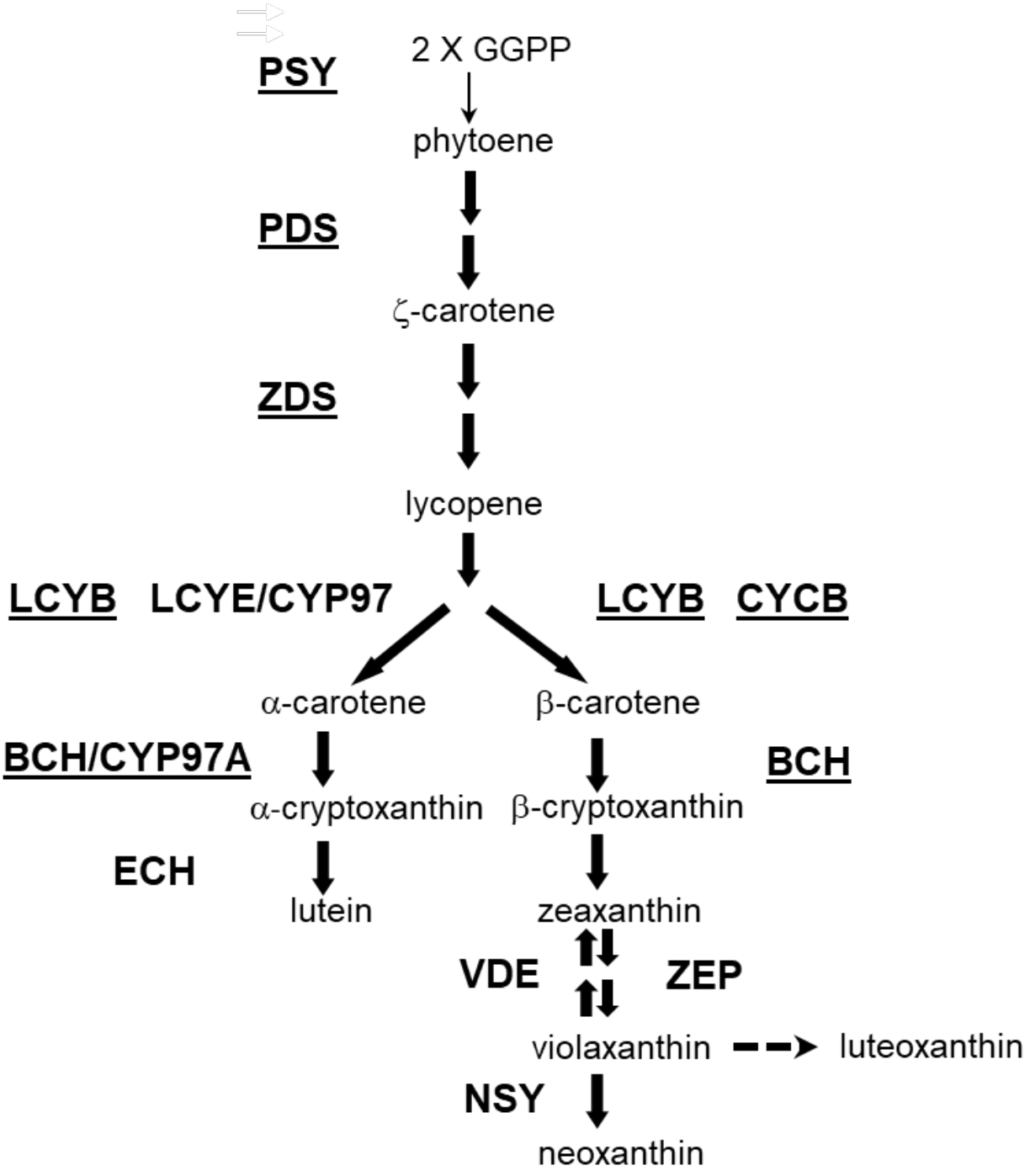
699 **Figure 4.** Relative expression of genes involved in carotenoid biosynthesis in peel (black bars)
700 and pulp (grey bars) of loquat fruit cv. Algerie (*Eriobotrya japonica* Lindl.) at breaker, yellow
701 and full color stages (BK, Y, FC). These ripening stages correspond to the stage 801, 803 and

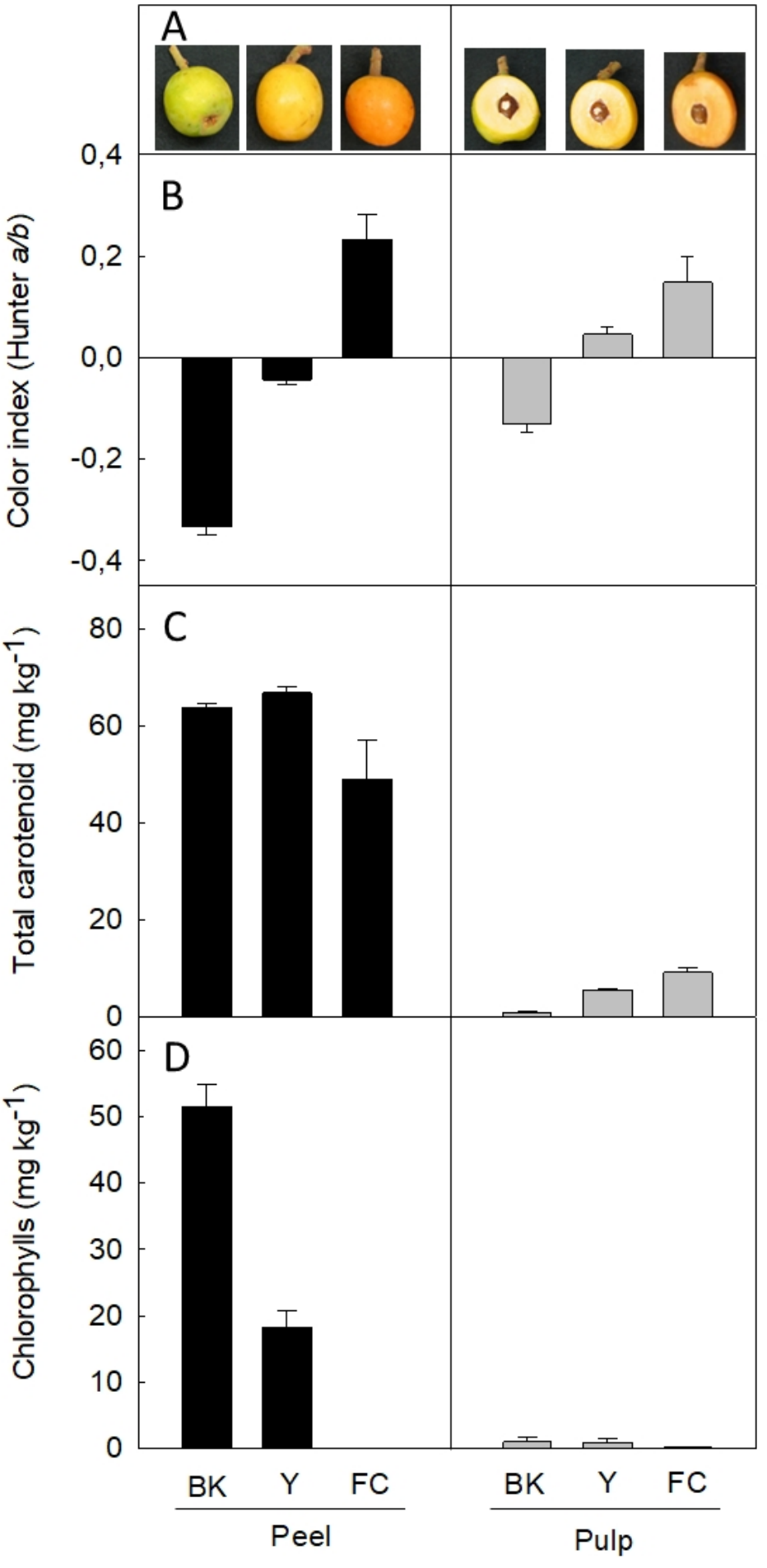
809 of the BBCH-scale. The genes measured were: *PSY, PDS, ZDS. CYCB* and *BCH*. The plots were arranged following the carotenoid biosynthetic sequence in the pathway. An expression value of 1 was arbitrarily assigned to the values obtained in the pulp of fruits at yellow stage. The data are means ± S.E of at least 3 replicates. Within each tissue, different letters for a given gene indicate statistically significant differences ($P \leq 0.05$).

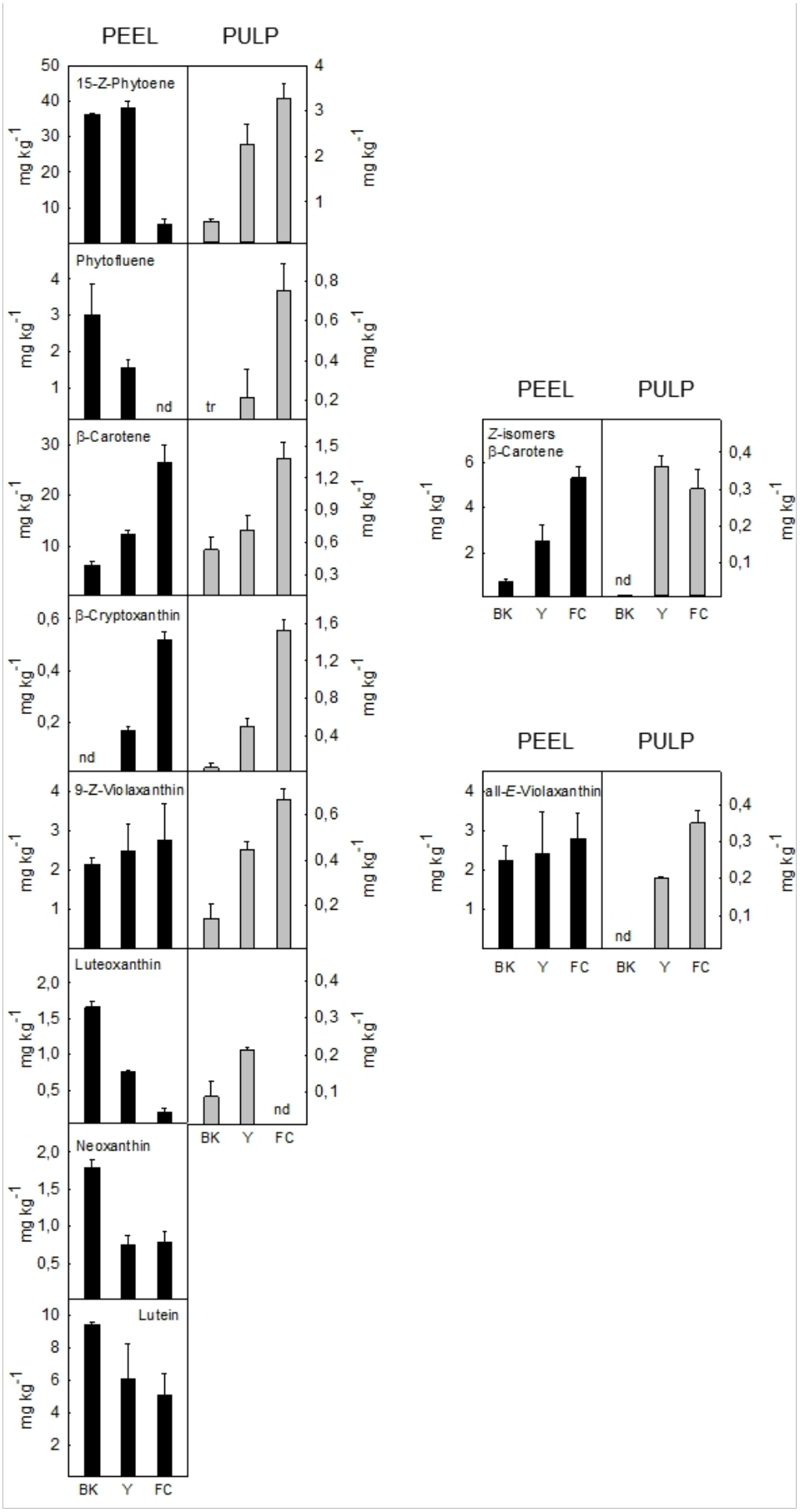
Figure 5. Effect of ethylene (ET, 10 µl L^{-1}) and 1-MCP (MCP, 1 µl L^{-1}) on color index (expressed as Hunter *a/b*) of peel (black bars) and pulp (grey bars) of loquat fruit cv. Algerie (*Eriobotrya japonica* Lindl.) harvested at breaker (A), yellow (B) and full color (C). Pictures of the fruits were taken at the beginning of the experiment (left) and after 6 days of incubation in air, ethylene or 1-MCP (right). Data are the mean ± S.E of at least 20 replicates.

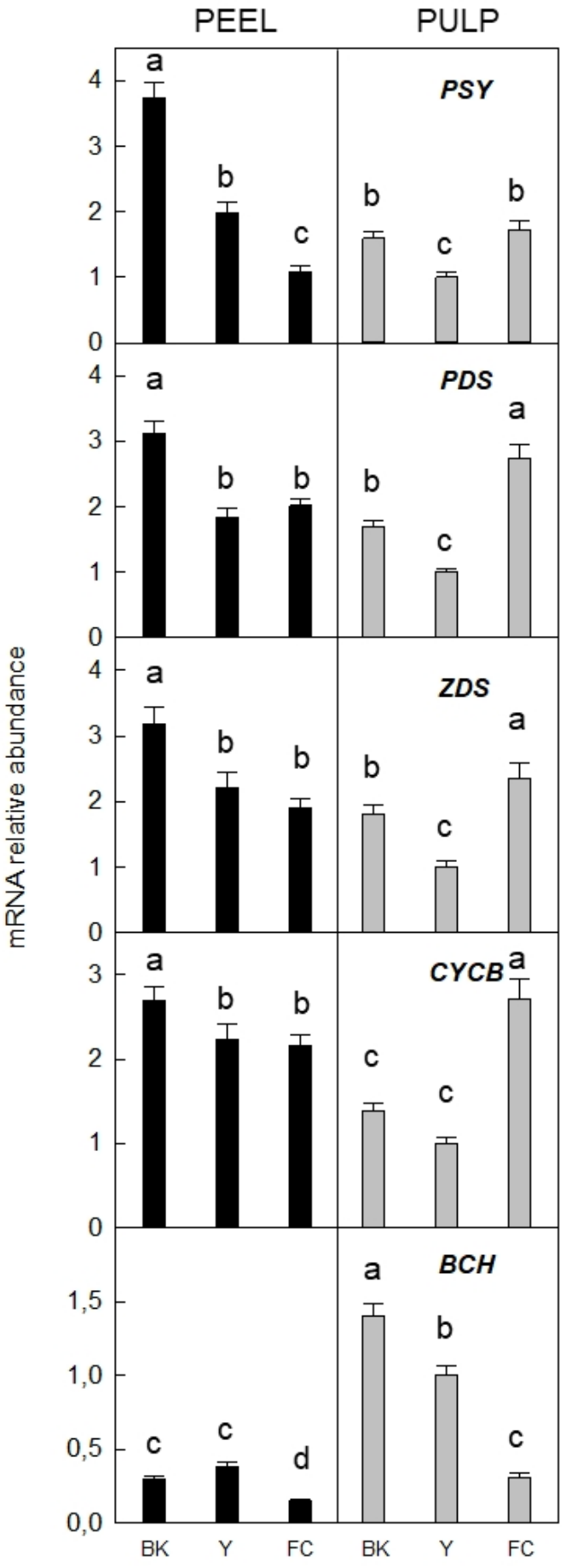
Figure 6. Effect of ethylene (ET, 10 µl L^{-1}) and 1-MCP (MCP, 1 µl L^{-1}) on the carotenoid content of peel (A) and pulp (B) of loquat fruit cv. Algerie (*Eriobotrya japonica* Lindl.) expressed as mg kg^{-1}. Fruits were treated at the yellow stage, corresponding to 803 of the BBCH-scale, and measurements were made at the onset of the experiment (day 0, 0d) and 2 (2d) and 6 (6d) days after treatment. The data are means ± S.E of at least 3 replicates.

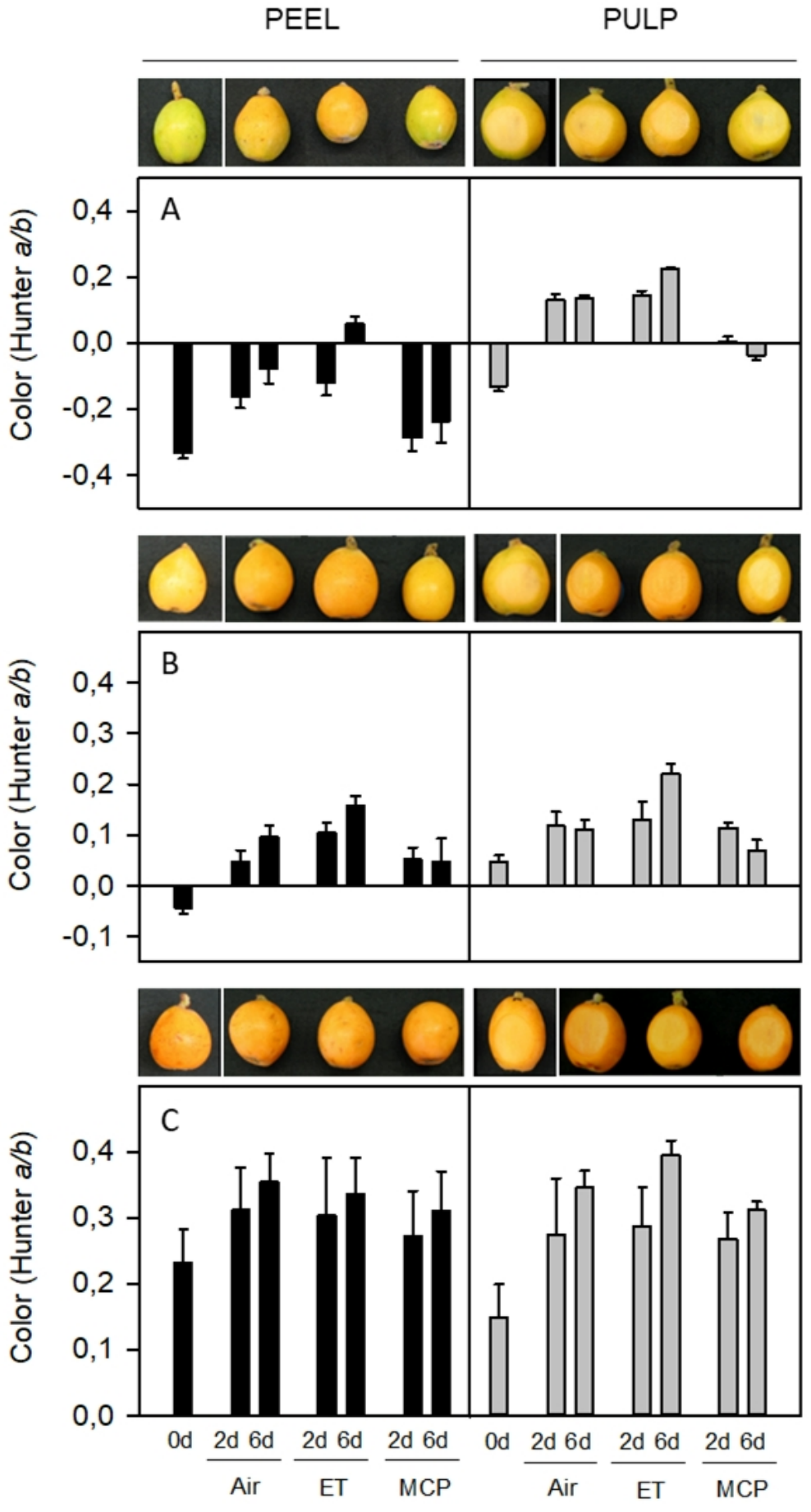
Figure 7. Effect of ethylene (ET, 10 µl L^{-1}) and 1-MCP (MCP, 1 µl L^{-1}) on the expression of carotenoid biosynthetic genes in peel (black bars) and pulp (grey bars) of loquat fruit cv. Algerie (*Eriobotrya japonica* Lindl.). Fruits were treated at the yellow stage, corresponding to 803 of the BBCH-scale and measurements were made the onset of the experiment (day 0, 0d) and after 2 (2d) and 6 days (6d) after the treatment. Expression of the following genes was determined: *PSY, PDS, ZDS, CYCB* and *BCH*. The plots were arranged following the carotenoid biosynthetic sequence in the pathway. The data are means ± S.E of at least 3 replicates. Within each tissue, different letters for a given gene indicate statistically significant differences ($P \leq 0.05$).

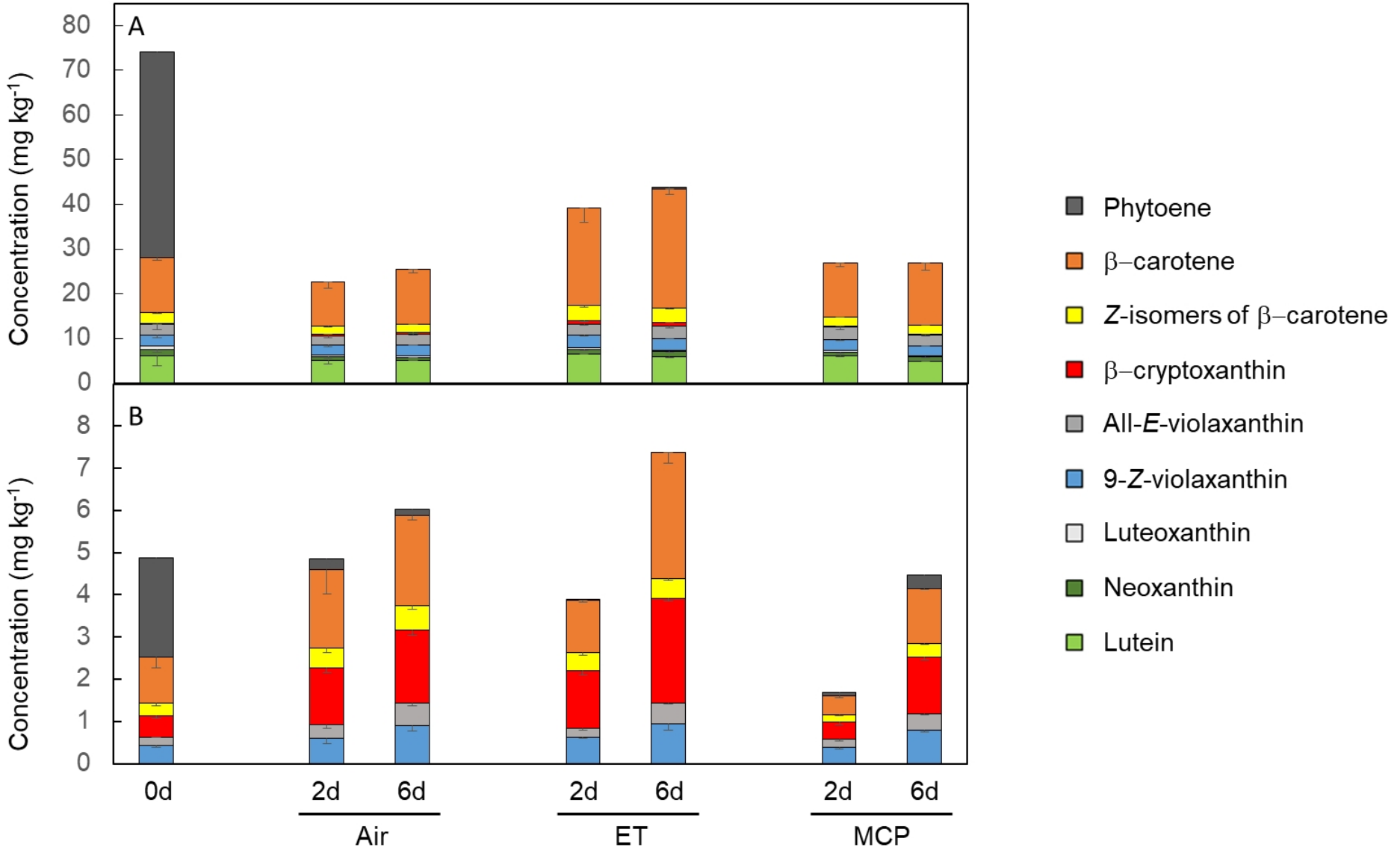

2 X GGPP
PSY
phytoene
PDS
ζ-carotene
ZDS
lycopene
LCYB
LCYE/CYP97
LCYB
CYCB
α-carotene
β-carotene
BCH/CYP97A
BCH
α-cryptoxanthin
β-cryptoxanthin
ECH
lutein
zeaxanthin
VDE
ZEP
violaxanthin
luteoxanthin
NSY
neoxanthin

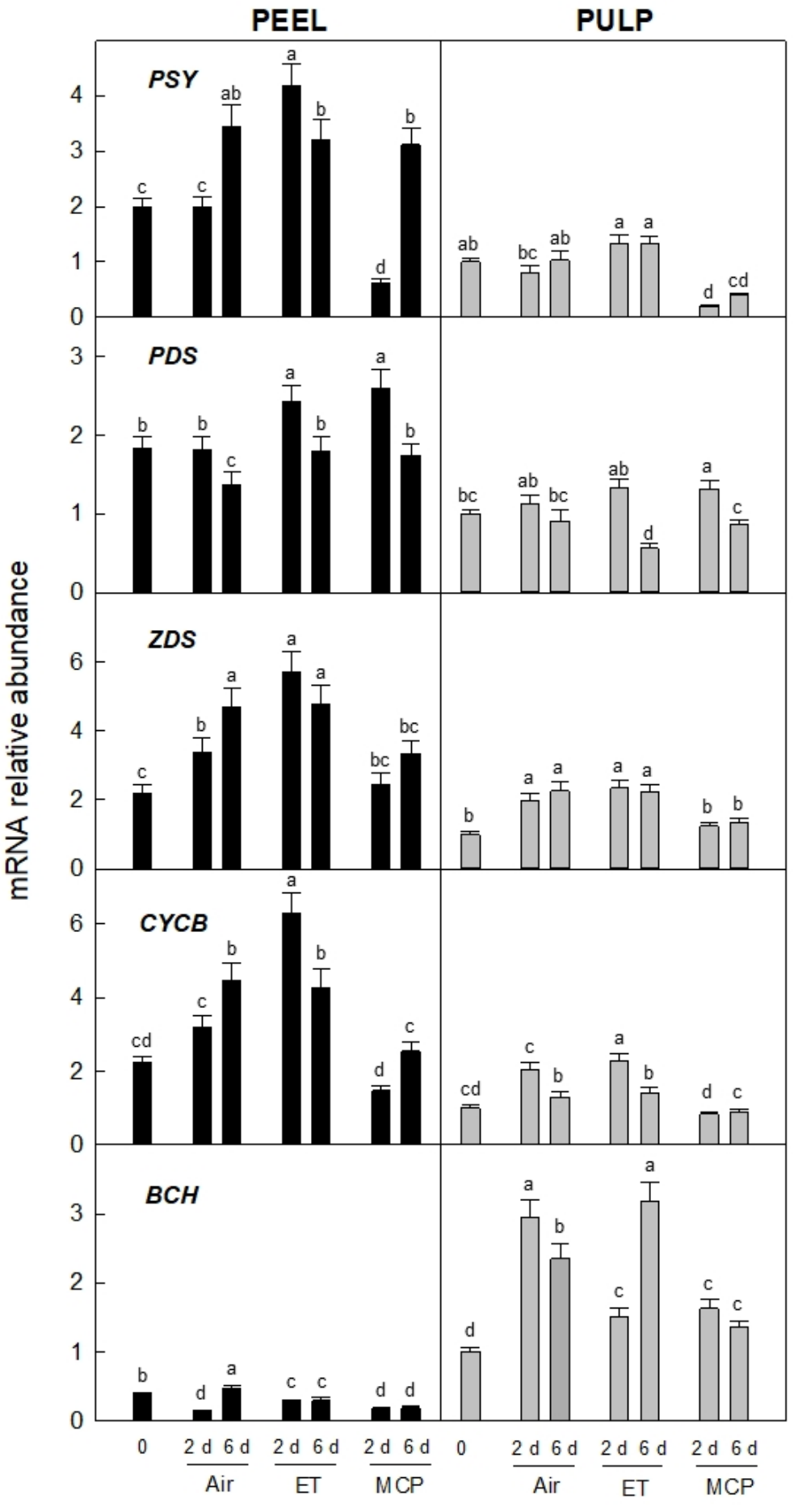

A
B
C
D
Color index (Hunter a/b)
0,4
0,2
0,0
-0,2
-0,4
Total carotenoid (mg kg-1)
80
60
40
20
0
Chlorophylls (mg kg-1)
60
50
40
30
20
10
0
BK
Y
FC
BK
Y
FC
Peel
Pulp


PEEL
PULP
15-Z-Phytoene
Phytofluene
β-Carotene
β-Cryptoxanthin
9-Z-Violaxanthin
Luteoxanthin
Neoxanthin
Lutein
Z-isomers β-Carotene
all-E-Violaxanthin
nd
tr
BK
Y
FC
mg kg-1


PEEL
PULP
mRNA relative abundance
PSY
PDS
ZDS
CYCB
BCH
BK
Y
FC
BK
Y
FC


PEEL
PULP
A
B
C
Color (Hunter a/b)
0,4
0,2
0,0
-0,2
-0,4
0,3
0,1
-0,1
0d
2d 6d
Air
ET
MCP


A
B
Concentration (mg kg-1)
Concentration (mg kg-1)
0
10
20
30
40
50
60
70
80
0
1
2
3
4
5
6
7
8
0d
2d
6d
2d
6d
2d
6d
Air
ET
MCP
Phytoene
β−carotene
Z-isomers of β−carotene
β−cryptoxanthin
All-E-violaxanthin
9-Z-violaxanthin
Luteoxanthin
Neoxanthin
Lutein


PEEL
PULP
PSY
PDS
ZDS
CYCB
BCH
mRNA relative abundance
0
2 d
6 d
Air
ET
MCP