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Abbreviations

ABA abscisic acid

C-neo 9'-*cis*-neoxanthin

C-vio 9-*cis*-violaxanthin

GA gibberellin

GGPP geranylgeranyl diphosphate

HYb β-ring hydroxylase

HYe ε-ring hydroxylase

LCY lycopene cyclase

LCYb lycopene β-cyclase

LCYe lycopene ε-cyclase

Lut lutein

NAA naphthalene acetic acid

NCED 9-*cis*-epoxycarotenoid dioxygenase

PDS phytoene desaturase

PSY phytoene by phytoene synthase

ZDS ζ-carotene desaturase

Zea zeaxanthin

α-Car α-carotene

β-Car β-carotene

β-Cry β-cryptoxanthin

Abstract

In the present study, to investigate the mechanisms regulating carotenoid accumulation in citrus, we set up a culture system with juice sacs of three citrus varieties, Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*Citrus sinensis* Osbeck) and Lisbon lemon (*Citrus limon* Burm.f.) *in vitro*. The juice sacs of all the three varieties enlarged gradually with carotenoid accumulation. The changing patterns of carotenoid content and the expression of carotenoid metabolic genes in juice sacs *in vitro* were similar to those ripening on trees in the three varieties. Using this system, the changes in the carotenoid content and the expression of carotenoid metabolic genes in response to environmental stimuli were investigated. The results showed that carotenoid accumulation was induced by blue light treatment, but not affected by red light treatment in the three varieties. Different regulation of *CitPSY* expression, which was up-regulated by blue light, while unaffected by red light, led to different changes in carotenoid content in response to these two treatments in Satsuma mandarin and Valencia orange. In all three varieties, increases in carotenoid content were observed in sucrose and mannitol treatments. However, the accumulation of carotenoid in the two treatments was regulated by distinct mechanisms at the transcriptional level. With ABA treatment, gene expression investigated in this study was up-regulated in Satsuma mandarin and Lisbon lemon, indicating that ABA induced its own biosynthesis at the transcriptional level. This feedback

77 regulation of ABA led to decreases in carotenoid content. With GA treatment,
78 carotenoid content were significantly decreased in the three varieties. Changes
79 in the expression of genes related to carotenoid metabolism varied among the
80 three varieties in response to GA treatment. These results provided insights into
81 improving carotenoid content and composition in citrus during fruit maturation.

82

83 **Key words:** Carotenoid, citrus, *in vitro*, juice sacs, regulatory mechanism.

Introduction

Carotenoids, which are important natural isoprenoid pigments, fulfill a variety of critical functions in plants, such as the stabilization of lipid membranes, light harvesting for photosynthesis, as well as protecting the photosystem from photo-oxidation (Havaux, 1998; Ledford and Niyogi, 2005). In addition, carotenoids are also precursors of the plant hormone abscisic acid (ABA), and exploited as coloring agents in flowers and fruits to attract pollinators (Schwartz *et al.*, 1997; Cunningham and Gantt, 1998). Carotenoids are important not only to the plants that produce them, but also to animals and humans. Some carotenoids containing β–ring moieties are the precursors of vitamin A, and have been proven to prevent the onset of certain chronic diseases and cancers (Giovannucci, 1999; Krinsky *et al.*, 2003). In citrus, carotenoids are the pigments responsible for the external and internal coloration of the fruits, and their contents and compositions are important indexes for the commercial and nutritional quality of the fruits. Carotenoid content and composition are influenced by growing conditions, geographical origins and fruit maturity; therefore they vary greatly among citrus varieties. In Satsuma mandarin (*Citrus unshiu* Marc.), β-cryptoxanthin (β-cry) is accumulated predominantly in juice sacs (Goodner *et al.*, 2001; Kato *et al.*, 2004). In contrast, Valencia orange (*Citrus sinensis* Osbeck) mainly accumulates violaxanthin (vio) isomers with 9-*cis*-violaxanthin (c-vio) as the principal carotenoid (Molnár and Szabolcs, 1980; Lee and Castle, 2001). Lisbon lemon (*Citrus limon* Burm.f.) also

106 accumulates β-cry as the principal carotenoid, but it accumulates much lower
107 level of carotenoid than Satsuma mandarin and Valencia orange. These citrus
108 varieties, which exhibit different carotenoid profiles, are useful for investigating
109 the mechanism of carotenoid accumulation. In the previous studies, the
110 relationship between carotenoid accumulation and expression of genes related to
111 carotenoid metabolism in different citrus varieties were investigated during
112 natural ripening (Kato *et al.*, 2004, 2006; Alquézar *et al.*, 2009).

113 Carotenoid metabolism has been well documented in various plant species,
114 including Arabidopsis (Park *et al.*, 2002), tomato (Isaacson *et al.*, 2002), pepper
115 (Bouvier *et al.*, 1998), tobacco (Busch *et al.*, 2002), alga (Steinbrenner and
116 Linden, 2001), citrus (Kato *et al.*, 2004; Rodrigo *et al.*, 2004; Rodrigo and
117 Zacarías, 2007), and apricot (Marty *et al.*, 2005; Kita *et al.*, 2007). As shown in
118 Fig. 1, the pathway of carotenoid metabolism in plants is a series of desaturation,
119 cyclization, hydroxylation, and epoxidation steps (Cunningham and Gantt, 1998;
120 Kato *et al.*, 2004). The conversion of geranylgeranyl diphosphate (GGPP) to
121 phytoene by phytoene synthase (PSY) is the first and rate-limiting step in the
122 pathway. Two functionally similar enzymes, phytoene desaturase (PDS) and
123 ζ-carotene desaturase (ZDS), convert phytoene to lycopene via phytofluene,
124 ζ-carotene and neuroporene. The cyclization of lycopene catalyzed by lycopene
125 cyclase (LCY) is a key branch point in the pathway in citrus fruits, yielding
126 α-carotene (α-car) and β-carotene (β-car). The genes of lycopene β-cyclase
127 (LCYb) and lycopene ε-cyclase (LCYe) have been identified in citrus (Kato *et*

al., 2004). α-Car is converted to lutein (lut), a major xanthophyll, by ε-ring hydroxylase (HYe) and β-ring hydroxylase (HYb). β-Car is converted to zeaxanthin (zea) via β-cry by a two-step hydroxylation, which is catalyzed by HYb, then zea is converted to vio by zea expoxidase (ZEP). In addition, carotenoid metabolism is closely related to the biosynthesis of plant hormones: abscisic acid (ABA) and gibberellin (GA). In higher plants, ABA is biosynthesized by the oxidative cleavage of certain xanthophylls. 9-*Cis*-epoxycarotenoid dioxygenases (NCED) catalyze the cleavage of 9-*cis*-violaxanthin (c-vio) or 9'-*cis*-neoxanthin (c-neo) to form C_{25} epoxy-apocarotenal and xanthoxin (C_{15}), from which the latter is the direct precursor of ABA. Similar to ABA, GA is also in close association with the biosynthesis of carotenoids. Like in the initial reaction of the carotenoid biosynthesis, GGPP is also the substrate for *ent*-copalyl diphosphate synthase, which together with *ent*-kaurene synthase lead to the metabolic flux into the biosynthesis of GA.

Recently, genes encoding enzymes for the main steps of carotenoid metabolism have been isolated and their expression has been characterized in plants (Kato *et al.*, 2004, 2006; Kita *et al.*, 2007; Alquézar *et al.*, 2009). During fruit ripening, transcriptional regulation of carotenoid genes appears to be a major mechanism by which biosynthesis and accumulation of specific carotenoids are regulated. In tomato, increases in the gene expression of *PSY* and *PDS*, and decreases in the gene expression of *LCYb* and *LCYe* led to the

accumulation of lycopene during fruit repining (Pecker *et al.*, 1996; Ronen *et al.*, 1999). In our previous studies, we found that as fruit maturation progressed, a simultaneous increase in the expression of genes (*CitPSY*, C*itPDS*, *CitZDS*, *CitLCYb*, *CitHYb* and *CitZEP*) led to massive β,β-xanthophll (β-cry, zea and vio) accumulation in the flavedo and juice sacs of Satsuma mandarin and Valencia orange (Kato *et al.*, 2004). Meanwhile, the gene expression of *CitNCED2* and *CitNCED3* in Satsuma mandarin and the gene expression of *CitNCED2* in Lisbon lemon were primarily responsible for the accumulation of ABA in juice sacs, while in Valencia orange the extremely low level of *CitNCED2* was primarily responsible for the low level of ABA (Kato *et al.*, 2006).

Carotenoid metabolism is a complicated process, which is regulated throughout the life cycle of a plant with dynamic changes in content and composition in response to environmental stimuli (Cazzonelli and Pogson, 2010). Light and sugar have been reported to be important environmental factors regulating carotenoid metabolism in plants (Huff, 1983, 1984; Alba *et al.*, 2000; Domingo *et al.*, 2001; Schofield and Paliyath, 2005; Wu *et al.*, 2007; Liu *et al.*, 2009). Additionally, plant hormones ABA and GA, which are closely involved in carotenoid metabolism, also play a crucial role in adjusting carotenoid content and composition in plants (Wan and Li, 2006; Rodrigo and Zacarías, 2007). To date, however, although significant advances have been made in understanding the accumulation of carotenoid and the expression of carotenoid metabolic genes during the maturation of citrus fruits, information about the changes in

carotenoid metabolism in response to various environmental stimuli in citrus fruits is still limited (Rodrigo and Zacarías, 2007; Matsumoto *et al.*, 2009). The tissue culture technique is one of the key tools to study plants growth and development, by which undefined variables were minimized and medium compositions and environmental factors were carefully controlled. So far, several attempts have been performed to culture citrus *in vitro* using different plant tissues (Mukai *et al.*, 2000; Harada *et al.*, 2001; Khan *et al.*, 2009). In the present study, to further investigate how the carotenoid accumulation is regulated in response to different environmental factors in citrus, we set up a culture system with juice sacs of three different citrus varieties, Satsuma mandarin, Valencia orange and Lisbon lemon. The juice sacs of the three varieties grew with carotenoid accumulation, and no callus formed throughout the experimental period. Using this system, the effects of environmental conditions (blue and red LED lights, sucrose and mannitol) and plant hormones (ABA and GA) on carotenoid content and composition, and the gene expression related to carotenoid biosynthesis and catabolism were investigated in the three varieties, Satsuma mandarin, Valencia orange and Lisbon lemon *in vitro*. This study gave more information on how carotenoid accumulation is regulated, which might provide new strategies to enhance carotenoid production in citrus.

Materials and methods

Plant materials

194 Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*Citrus sinensis*
195 Osbeck) and Lisbon lemon (*Citrus limon* Burm.f.) cultivated at the National
196 Institute of Fruit Tree Science, Department of Citrus Research, Okitsu (Shizuoka,
197 Japan) were used as materials.

198 *In vitro culture system*

199 The fruits were surface-sterilized by a 10-min soak in 70% ethanol, a 30-min
200 soak in 1% (w/v) NaOCl, and rinsed in sterile water. Juice sacs were excised
201 from the equatorial region of the fruit, were placed on 10 ml of agar medium in
202 culture tubes (22 × 120 mm) and incubated in the dark at 25 °C. The explants
203 were placed with the endocarp side up, so that the juice sacs were not in contact
204 with the Murashige and Skoog (MS) medium supplemented with 10% (w/v)
205 sucrose and 1% (w/v) agar. The pH of the MS medium was adjusted to 5.7 and
206 autoclaved. The explants were taken out of their tubes and the carotenoid
207 content was determined every two weeks. The juice sacs were immediately
208 frozen in liquid nitrogen, and kept at –80 °C until use.

209 *Extraction and determination of ascorbic acid*

210 The ascorbic acid content was assayed by HPLC. Each frozen sample was
211 homogenized using a mortar and pestle in 10 volumes of extractant solution (3%
212 metaphosphoric acid and 8% acetic acid). The homogenate was centrifuged at
213 14,000×g for 20 min, and then the supernatant was filtered through Miracloth
214 (Calbiochem). The pH of the filtrate was adjusted by adding an equal volume of
215 0.2 M potassium-phosphate buffer (pH 7.5). The total ascorbic acid was assayed

by adding 0.5 ml of 6 mM dithiothreitol (DTT) to 0.1 ml of aliquot of filtrate and incubated in the dark at 30 °C for 15 min. After the sample was filtered through a 0.22-μm cellulose acetate filter (Advantec), a 20 μl aliquot was injected onto a J'sphere ODS-M80 column (YMC) attached to a LC-10AD pump (Shimadzu). The column kept at 20 °C was eluted with 1.5% ammonium dihydrogen phosphate (pH 3.8) at a flow rate of 1.0 ml min^{-1}. The ascorbic acid content was monitored at 245 nm (retention time 2.6 min) using an SPD-10A spectrophotometric detector (Shimadzu) attached to a chart recorder (C-R6A, Shimadzu). Peaks were converted to concentrations by using the dilution of stock ascorbic to construct a standard curve.

Treatments

The juice sacs were cultured for two weeks under the same conditions as described above, and then irradiated with blue (peak wavelength, 470 nm) and red (peak wavelength, 660 nm) LED lights at an intensity of 50 μmol $m^{-2}s^{-1}$ for two weeks. For the sucrose and mannitol treatments, the explants with the endocarp side up were placed on MS medium supplemented with 15% (w/v) sucrose or 6% (w/v) mannitol for four weeks. For the ABA and GA treatments, the explants with the endocarp side up were placed on MS medium supplemented with ABA (10 μM) and GA_3 (10 μM) for four weeks. Juice sacs cultured in the dark for four weeks were used as the control. After each treatment, the juice sacs were immediately frozen in liquid nitrogen, and kept at –80 °C until use.

Extraction and determination of carotenoids

The identification, extraction and quantification of carotenoid in citrus have been described previously (Kato *et al.*, 2004). β-Car, β-cry, all-*trans*-violaxanthin (t-vio), c-vio and lut were quantified in the juice sacs of Satsuma mandarin, Valencia orange and Lisbon lemon during the experimental period. The contents of carotenoids were expressed as μg g^{-1} fresh weight. Carotenoid quantification was performed in three replicates.

Total RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from the juice sacs of Satsuma mandarin, Valencia orange and Lisbon lemon fruits at different stages according to the method described by Ikoma *et al.* (1996). The total RNA was cleaned up with the RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase digestion. The reactions of reverse transcription (RT) were performed with 2 μg of purified RNA and a random hexamer at 37 ˚C for 60 min using TaqMan Reverse Transcription Reagents (Applied Biosystems).

TaqMan MGB probes and sets of primers for *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, *CitZEP*, *CitNCED2* and *CitNCED3* were designed on the basis of the common sequences among the three varieties for each gene with the Primer Express software (Applied Biosystems; Kato *et al.*, 2007; Alquézar *et al.*, 2009). For endogenous control, the TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems) was used. TaqMan real-time PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using

ABI PRISM 7300 (Applied Biosystems) according to the manufacture's instructions. Each reaction contained 900 nM primers, a 250 nM TaqMan MGB Probe, and template cDNA. The thermal cycling conditions were 95 ˚C for 10 min followed by 40 cycles of 95 ˚C for 15 s and 60 ˚C for 60 s. The levels of gene expression were analyzed with ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems) and normalized with the results of 18S ribosomal RNA. Real-time quantitative RT-PCR was performed in three replicates for each sample.

Statistical analysis

All values are shown as the mean ± SE for three replicates. The data were analyzed, and Tukey's HSD test was used to compare the means at $P < 0.05$.

Results

Tissue culture of citrus juice sacs in vitro

The juice sacs of Satsuma mandarin, Valencia orange and Lisbon lemon were cultured *in vitro* for eight weeks. As shown in Fig. 2, the juice sacs of all three varieties enlarged rapidly without the formation of callus throughout the experimental period. In Satsuma mandarin and Valencia orange the juice sacs turned yellow gradually, while in Lisbon lemon the changes in the color of juice sacs were less obvious during the experimental period (Fig. 2).

Changes in the carotenoid content and the gene expression related to carotenoid metabolism

Changes in the content and composition of carotenoid were examined every two weeks. Massive accumulation of carotenoids, especially β,β-xanthophylls, occurred in Satsuma mandarin and Valencia orange during the experimental period (Fig. 3, A and B). In Satsuma mandarin, the contents of β-cry, t-vio and c-vio increased rapidly along with the total carotenoid accumulation throughout the experimental period. In Valencia orange, the contents of t-vio and c-vio increased significantly in the first four weeks. In Lisbon lemon, the total carotenoid content remained extremely low, although β-cry accumulated gradually throughout the experimental period (Fig. 3C). The content of lut, a major β,ε-carotenoid, increased clearly in the first two weeks and then remained constant in the three varieties.

The expression of a set of genes to produce β,β-xanthophylls (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb* and *CitZEP*) increased from the second week after cultured *in vitro* in Satsuma mandarin and Valencia orange. Moreover, the gene expression levels of *CitZDS*, *CitLCYb1* and *CitLCYb2* were higher in Satsuma mandarin than those in Valencia orange. In contrast, the gene expression levels of *CitHYb* and *CitZEP* were much higher in Valencia orange than those in Satsuma mandarin. In Lisbon lemon, the expression of a set of genes that produce β,β-xanthophylls, increased slightly from the sixth week after cultured *in vitro*. However, the gene expression levels of *CitPSY*, *CitZDS*, *CitLCYb2*, *CitHYb* and *CitZEP* were much lower than those in Satsuma mandarin and Valencia orange (Fig. 4).

Effects of blue and red LED lights on carotenoid content and gene expression related to carotenoid metabolism

With the treatment of blue light, the accumulations of β-cry, t-vio and lut were induced significantly along with an increase in the total carotenoid content in Satsuma mandarin (Fig. 5A). In Valencia orange, the contents of β-car, t-vio and lut were increased by the treatment with blue light, as a result the total carotenoid content was much higher than that in the control (Fig. 5B). In Lisbon lemon, the three carotenoids detected in this study (β-car, β-cry and lut) were clearly increased by the blue light treatment (Fig. 5C). In contrast to blue light treatment, red light treatment had no obvious effects on the contents of the carotenoids investigated in the present study in Satsuma mandarin and Valencia orange. In Lisbon lemon, the red light treatment induced a slight increase in β-cry, while it did not affect the contents of other carotenoids.

As shown in Fig. 6, in Satsuma mandarin, the gene expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitHYb*, *CitZEP* and *CitNCED3* was up-regulated simultaneously by blue light (Fig. 6A). In Valencia orange, the expression of genes investigated in the present study was up-regulated by blue light, except for *CitNCED3* (Fig. 6B). In Lisbon lemon, the induction of *CitPSY*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitZEP* and *CitNCED3* was observed in the blue light treatment (Fig. 6C).

Red light treatment did not affect the gene expression of *CitPSY*, *CitPDS*, *CitZDS* or *CitLCYb1*, while slightly increased the gene expression of *CitHYb*,

326 *CitZEP*, *CitNCED2* and *CitNCED3* in Satsuma mandarin (Fig. 6A). Similar to
327 Satsuma mandarin, in Valencia orange, noticeable increase in the gene
328 expression of *CitPSY*, *CitPDS*, *CitZDS* was not observed in the red light
329 treatment. The gene expression of *CitLCYb1*, *CitLCYb2*, *CitNCED2* and
330 *CitNCED3* was up-regulated in red light-treated Valencia orange (Fig. 6B). In
331 Lisbon lemon, the expression of the genes investigated in the present study was
332 up-regulated by the red light treatment, except for *CitHYb* (Fig. 6C).

333 *Effects of sucrose and mannitol on carotenoid content and gene expression*
334 *related to carotenoid metabolism*

335 The treatments with sucrose and mannitol induced the accumulation of
336 carotenoids in the juice sacs of Satsuma mandarin, Valencia orange and Lisbon
337 lemon (Fig. 7). In Satsuma mandarin and Valencia orange, the contents of
338 carotenoids investigated in the present study were simultaneously increased by
339 the treatments with sucrose and mannitol. In Lisbon lemon, β-car and β-cry
340 contents were increased by the treatments with sucrose and mannitol. The
341 content of lut was increased by the mannitol treatment, while it was not
342 significantly affected by the sucrose treatment in Lisbon lemon.

343 In Satsuma mandarin, the gene expression of *CitPSY*, *CitZDS*, *CitNCED2* and
344 *CitNCED3* was up-regulated, while the gene expression of *CitLCYb2*, *CitHYb*
345 and *CitZEP* was down-regulated by the treatment with sucrose. In Valencia
346 orange, the gene expression of *CitPSY*, *CitNCED2* and *CitNCED3* was
347 up-regulated, while the gene expression of *CitPDS*, *CitZDS*, *CitLCYb1*,

CitLCYb2, *CitHYb* and *CitZEP* was down-regulated by the treatment with sucrose. In Lisbon lemon, the gene expression of *CitPSY*, *CitZDS*, *CitNCED2* and *CitNCED3* was up-regulated by the sucrose treatment.

With the treatment of mannitol, the gene expression of *CitPSY*, *CitZDS* and *CitZEP* was slightly down-regulated, while the gene expression of *CitLCYb2*, *CitHYb*, *CitNCED2* and *CitNCED3* was up-regulated in Satsuma mandarin (Fig. 8A). In Valencia orange, the gene expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitHYb* and *CitZEP* was down-regulated, while the gene expression of *CitLCYb2*, *CitNCED2* and *CitNCED3* was up-regulated by mannitol treatment (Fig. 8B). In Lisbon lemon, the up-regulation of gene expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitNCED2* and *CitNCED3*, and the down-regulation of gene expression of *CitHYb* and *CitZEP* were observed in the mannitol treatment (Fig. 8C).

Effects of ABA and GA on carotenoid content and gene expression related to carotenoid metabolism

In Satsuma mandarin, the contents of t-vio, c-vio and lut were increased slightly by the treatment with ABA. While the content of β-cry, the prominent carotenoid accumulated in Satsuma mandarin, was decreased significantly along with a decrease in the total carotenoid content by ABA treatment. With GA treatment, the contents of β-cry, t-vio, c-vio and lut were simultaneously decreased, and as a result the total carotenoid content was much lower than that of the control (Fig. 9A). In Valencia orange, the contents of β-car, t-vio, c-vio

and lut were decreased significantly by the treatments with ABA and GA (Fig. 9B). In Lisbon lemon, the contents of total carotenoid, β-car and lut were decreased by ABA and GA treatments, while β-cry content was not affected by the two treatments (Fig. 9C).

In Satsuma mandarin and Lisbon lemon, the expression of genes detected in the present study (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, *CitZEP*, *CitNCED2* and *CitNCED3*) was simultaneously up-regulated by the treatment with ABA (Fig. 10, A and C). In Valencia orange, the gene expression of *CitPSY*, *CitPDS*, *CitLCYb1* and *CitHYb* was up-regulated, while the gene expression of *CitNCED2* and *CitNCED3* was down-regulated by the treatment with ABA (Fig. 10B).

With the treatment of GA, the gene expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitHYb*, *CitZEP* and *CitNCED2* was up-regulated, while the gene expression of *CitLCYb1*, *CitLCYb2* and *CitNCED3* was down-regulated in Satsuma mandarin. In Valencia orange, the expression of the genes investigated in this study was simultaneously down-regulated by the treatment with GA, except for *CitLCYb1*. In Lisbon lemon, the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitHYb*, *CitZEP* and *CitNCED2* was up-regulated, while the expression of *CitLCYb2* was not affected by the treatment with GA (Fig. 10).

Discussion

Carotenoid accumulation in vitro

Carotenoid metabolism is a complicated process in plants, which is affected

392 by developmental requirements and environmental stimuli (Cazzonelli and
393 Pogson, 2010). It is difficult to evaluate the effects of environmental stimuli on
394 carotenoid metabolism in the fruits ripening on trees, as the growing conditions
395 on trees are not uniform and hard to be controlled. In the present study, to
396 further investigate the regulation of carotenoid metabolism in citrus, we firstly
397 developed an *in vitro* system, in which undefined variables were minimized and
398 medium compositions and environmental factors were carefully controlled. In
399 this system, the juice sacs enlarged gradually with carotenoid accumulation and
400 no callus formed throughout the experimental period in the three citrus varieties,
401 Satsuma mandarin, Valencia orange and Lisbon lemon. In our previous study,
402 sugar accumulation in the juice sacs has been reported using the same culture
403 system (Mukai *et al*., 2000). This study showed that sugar contents gradually
404 increased until two months, and then increased rapidly. The pattern of sugar
405 accumulation was similar to that of juice sacs in field-grown fruits. After eight
406 weeks, the sugar content reached 4.01% in the juice sacs cultured *in vitro*, which
407 is similar to that in the intact fruits (3.91%). In the present study, the changes in
408 carotenoid contents were detected in the juice sacs of the three varieties cultured
409 *in vitro*. After eight weeks, in Satsuma mandarin and Lisbon lemon, the content
410 of β-cry, which is the predominantly accumulated carotenoid, reached 5.5 μg g^{-1}
411 and 0.13 μg g^{-1}, respectively. In Valencia orange, c-vio was abundant, which
412 increased significantly in the first four weeks, and reached 0.8 μg g^{-1} after eight
413 weeks. It has been reported that a change from β,ε-carotenoid accumulation to

β,β-xanthophylls accumulation occurred in the flavedo and juice sacs of citrus fruits during the ripening process (Kato *et al.*, 2004; Alquézar *et al.*, 2008). In this study, the accumulation of β,β-xanthophylls was observed in the three citrus varieties during the experimental period, whereas the content of lut, which is a major β,ε-carotenoid in citrus, clearly increased in the first two weeks and then remained constant. The changes in the carotenoid content and composition in the three citrus varieties cultured *in vitro* were similar to those in citrus fruits ripening on trees (Kato *et al.*, 2004; Alquézar *et al.*, 2008). In addition, the changes of ascorbic acid in the juice sacs cultured *in vitro* were also detected. During the experimental period, the ascorbic acid content kept constant at a lower level in Satsuma mandarin, while it decreased significantly in Valencia orange and Lisbon lemon (Table S1). The changes in the ascorbic acid content in the juice sacs cultured *in vitro* were similar to those in the intact fruits.

Transcriptional regulation of carotenoid genes is a major mechanism by which the biosynthesis and accumulation of specific carotenoids are regulated during fruit ripening (Kato *et al.*, 2004, 2006; Kita *et al.*, 2007; Alquézar *et al.*, 2009). In the present study, simultaneous increases in the gene expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb* and *CitZEP* were observed in Satsuma mandarin and Valencia orange. Compared with Satsuma mandarin and Valencia orange, the gene expression of *CitPSY*, *CitZDS*, *CitLCYb1*, *CitHYb* and *CitZEP* was much lower in Lisbon lemon. Additionally, the mRNA levels of *CitZDS*, *CitLCYb1* and *CitLCYb2* were higher in Satsuma

436 mandarin than those in Valencia orange. In contrast, the mRNA levels of *CitHYb*
437 and *CitZEP* were higher in Valencia orange than those in Satsuma mandarin.
438 The differences in the gene expression led to the differences in the
439 β,β-xanthophylls composition between Satsuma mandarin and Valencia orange.
440 The changing patterns of the gene expression in the three citrus varieties *in vitro*
441 were similar to those in the citrus fruits during nature ripening process (Kato *et*
442 *al.*, 2004). Therefore, in the present study we successfully set up a culture
443 system of citrus juice sacs *in vitro*, in which carotenoid metabolism in the juice
444 sacs was similar to that in the intact fruits. This system was useful to further
445 investigate the regulation of carotenoid metabolism by different environmental
446 factors in citrus fruits *in vitro*.

447 *Effects of blue and red LED lights on carotenoid metabolism*

448 In higher plants, sensing of light is carried out by various light photoreceptors
449 (Briggs, 2001). Thus, plants exhibit different responses to various lights. In the
450 present study, the results showed that total carotenoid content was increased by
451 the treatment with blue light (peak wavelength, 470 nm) in Satsuma mandarin,
452 Valencia orange and Lisbon lemon. Wu *et al.* (2007) reported that β-car content
453 was much higher in the red light-treated group than blue light-treated group in
454 leaves and stems of pea seedlings. In tomatoes, the accumulation of lycopene
455 along with an increase in total carotenoid content was also observed in response
456 to red light treatment (Schofield and Paliyath, 2005; Liu *et al.*, 2009). However,
457 our results showed that irradiation with red light (peak wavelength, 660 nm) did

458 not affect the content or composition of carotenoid in Satsuma mandarin and
459 Valencia orange. In Lisbon lemon, red light slightly increased the content of
460 β-cry, while the total carotenoid content was not significantly affected (Fig. 5).
461 These results indicated that regulatory effects of the blue and red lights on
462 carotenoid accumulation were cultivar-dependent, and in citrus blue light
463 treatment was more effective to induce carotenoid accumulation than red light.

464 PSY, which is a rate-limiting enzyme for carotenoid biosynthesis, is regulated
465 by light through a phytochrome-mediated process (von Lintig *et al.*, 1997; Alba
466 *et al.*, 2000). Bohne and Linden (2002) found that in *Chlamydomonas*
467 *reinhardtii* blue light was effective to up-regulate the gene expression of PSY,
468 whereas illumination with red light had no effects on the expression of this gene.
469 In the present study, we found that the gene expression of *CitPSY* was
470 up-regulated by the treatment with blue light in Satsuma mandarin, Valencia
471 orange and Lisbon lemon. The elevated expression of *CitPSY* was well
472 consistent with the accumulation of carotenoids in the three varieties treated
473 with blue light. In contrast to blue light, red light did not have significant effects
474 on the gene expression of *CitPSY* in Satsuma mandarin and Valencia orange.
475 Welsch *et al.* (2003) reported that the *cis*-acting elements in response to blue and
476 red lights were separated and located in different positions of the *PSY* promoter.
477 In Satsuma mandarin and Valencia orange, the difference in the regulation of
478 *CitPSY* in response to the blue and red lights might be related to the different
479 *cis*-acting elements for the two treatments in the *CitPSY* promoter.

Effects of sucrose and mannitol on carotenoid metabolism

In citrus fruits, sugar treatment promoted the accumulation of carotenoids and advanced the rate of color break, in which the color of the citrus peel changed from green to orange (Huff, 1983, 1984; Domingo et al. 2001). In the previous study, we found that sucrose (5%, 10% and 15%) and mannitol (0%, 3% and 6%) concentration-dependently induced the carotenoid accumulation in the juice sacs of citrus (data not shown). In the recent years, sugars are reported to act as primary messenger in signal transduction processes that trigger gene expression in plants and regulate many important processes (Foyer *et al*., 1997; Loreti *et al*., 2005). To date, however, it is still unknown how the carotenoid metabolism is regulated by sugar at the transcriptional level in citrus fruits. In the present study, the results showed that the gene expression of *CitPSY* simultaneously increased by sucrose treatment in Satsuma mandarin, Valencia orange and Lisbon lemon. The higher expression level of *CitPSY* contributed to the increases in the carotenoid contents in the sucrose treated samples of the three citrus varieties. In tomato fruit, increase in the expression of PSY was also observed in the sucrose treatment (Telef *et al*., 2006). In the treatment with mannitol, a simultaneous increase in the gene expression of *CitLCYb2* was observed in all three varieties. *LCYb2* is a key gene for the regulation the flux of carotenes into the β,β-branch of the pathway to lead to the increase of xanthophylls in citrus (Alquézar *et al*., 2009). In the mannitol treatment, the up-regulation of *CitLCYb2* contributed to the accumulation of carotenoids in the three varieties. These results suggested

502 that the sucrose- and mannitol-induced carotenoid accumulations were mediated
503 by regulating different steps of the carotenoid biosynthetic pathway in citrus
504 fruits.

505 In addition, the two carotenoid catabolic genes, *CitNCED2* and *CitNCED3*,
506 were up-regulated simultaneously by the sucrose and mannitol treatments in the
507 three citrus varieties. The expression of NCED, the rate-limiting enzyme for
508 ABA biosynthesis, is highly activated by stress conditions. Iuchi et al. (2000)
509 found that the induction of *VuNCED1* was mainly responsible for ABA
510 biosynthesis under water stress in cowpea. Increases in NCED genes expression
511 in response to drought stress were also observed in Arabidopsis, maize and
512 tomato (Burbidge *et al*., 1997; Schwartz *et al*., 1997; Qin and Zeevaart, 1999).
513 Sucrose and mannitol not only provide the common sources of carbon in tissue
514 cultures, but also might induce osmotic stress. Therefore, the up-regulation of
515 *CitNCED2* and *CitNCED3* in the three citrus varieties *in vitro* might be
516 attributed to the osmotic stress caused by sucrose and mannitol.

517 *Effects of plant hormones on carotenoid metabolism*

518 In higher plants, the biosynthesis of ABA, which is formed by the oxidative
519 cleavage of c-vio and c-neo, is involved in the carotenoid biosynthesis pathway
520 (Fig.1). As ABA and carotenoids share some steps in their biosynthesis
521 pathways, ABA level is closely related with carotenoids contents (Rodrigo *et al*.,
522 2003; Sarmad *et al*., 2007). In our previous study, we found that ABA
523 accumulation in Satsuma mandarin, Valencia orange, and Lisbon lemon

exhibited different changing patterns during fruit maturation, which indicated that the physiological role of ABA accumulation may be involved in the formation of different profiles of carotenoids in the three citrus varieties (Kato *et al.*, 2006). In this study, the content of total carotenoid was decreased clearly by the treatment with ABA in Satsuma mandarin, Valencia orange and Lisbon lemon (Fig. 9). The expression of the genes investigated in the present study (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, *CitZEP*, *CitNCED2* and *CitNCED3*) was simultaneously up-regulated by the treatment with ABA in Satsuma mandarin and Lisbon lemon, which indicated that ABA treatment induced its own biosynthesis at the transcriptional level in the two citrus varieties (Fig. 10). This positive feedback regulation of ABA led to decreases in the carotenoid content in Satsuma mandarin and Lisbon lemon. In Valencia orange the gene expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1* and *CitHYb* was up-regulated, while the gene expression of *CitNCED2* and *CitNCED3* was down-regulated by the treatment with ABA. The ABA content of Valencia orange was much lower than that of Satsuma mandarin and Lisbon lemon (Kato *et al.*, 2006). The extremely low level of ABA was closely related to the low mRNA level of *CitNCED2* in the juice sacs of Valencia orange. The differences in the regulation of NCED genes expression between the Valencia orange and the other two citrus varieties in response to ABA treatment might be attributed to the differences in the metabolism of ABA between Valencia orange and the other two varieties.

546 Similar to ABA, GA is also closely related to the biosynthesis of carotenoids
547 (Fig.1). It has been shown that treatment with GA has an important effect on
548 carotenoid metabolism by modification the early steps of the carotenoid
549 biosynthetic pathway (Iglesias *et al.*, 2001; Rodrigo and Zacarías, 2007, Zhou *et*
550 *al.*, 1996). The results in this paper showed that the total carotenoid content was
551 decreased by the treatment with GA in Satsuma mandarin, Valencia orange and
552 Lisbon lemon (Fig. 9). However, changes of gene expression varied among the
553 three varieties in response to GA treatment (Fig. 10). In the GA-treated Valencia
554 orange, the gene expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, *CitHYb* and
555 *CitZEP* was simultaneously down-regulated, which was well consistent with the
556 decrease in the carotenoid content. In Satsuma mandarin, the down-regulation of
557 gene expression of *CitLCYb1* and *CitLCYb2*, which were the key genes related
558 to the biosynthesis of xanthophylls, led to the decreases in the content of β-cry,
559 t-vio and c-vio in the treatment with GA. In Lisbon lemon, the gene expression
560 of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitHYb* and *CitZEP* was up-regulated by
561 the treatment with GA, which was not well consistent with the decrease in the
562 carotenoid content in GA-treated Lisbon lemon. Other regulatory mechanism,
563 such as post-transcriptional factors and other genes in the methyl erythritol
564 phosphate pathway (MEP), may also be involved in regulation of the carotenoid
565 content in Lisbon lemon in response to GA treatment.

566 In conclusion, carotenoid metabolism was investigated in response to
567 different environmental conditions (blue and red LED lights, sucrose and

mannitol) and plant hormones (ABA and GA) in three citrus varieties, Satsuma mandarin, Valencia orange and Lisbon lemon *in vitro*. The results showed that carotenoid accumulation was induced by the blue light, sucrose and mannitol treatments, while it was suppressed by the ABA and GA treatments in the three citrus varieties. The carotenoid metabolism in the three citrus varieties was not sensitive to the red light treatment, by which the total carotenoid content was not significantly affected. In addition, gene expression results showed that carotenoid metabolism in response to these treatments was highly regulated at the transcriptional level in Satsuma mandarin, Valencia orange and Lisbon lemon. The results presented here provide more insights into the regulatory mechanism of carotenoid metabolism in citrus, which might facilitate the improvement in carotenoid content and composition in citrus.

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Figure legends

Fig. 1. Carotenoid metabolic pathway in citrus. GGPP, geranylgeranyl diphosphate. The gene expression of the *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, *CitZEP*, *CitNCED2* and *CitNCED3* was analyzed by real-time PCR in this study.

Fig. 2. Changes in the appearance of juice sacs in the three citrus varieties during culture *in vitro*. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon.

Fig. 3. Changes in the carotenoid content in juice sacs of the three citrus varieties during culture *in vitro*. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon. Total car, total carotenoids. β-car, β-carotene. β-cry, β-cryptoxanthin. T-vio, all-trans-violaxanthin. C-vio, 9-*cis*-violaxanthin. Lut, lutein. The value for total carotenoids was the sum of identified carotenoids. Columns and bars represent the means and SE (n=3), respectively.

Fig. 4. Changes in the expression of carotenoid metabolism related genes in juice sacs of the three citrus varieties. The results shown are the mean ± SE for triplicate samples.

Fig. 5. Effect of blue and red LED lights on the carotenoid content in juice sacs of the three citrus varieties. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon. Total car, total carotenoids. β-car, β-carotene. β-cry, β-cryptoxanthin. T-vio, all-trans-violaxanthin. C-vio, 9-*cis*-violaxanthin. Lut, lutein. The value for total carotenoids was the sum of identified carotenoids. Columns and bars represent the means and SE (n=3), respectively. The letters a, b and c indicate significant differences among the control, blue light and red light treatments at the 5% level by Tukey's HSD test.

Fig. 6. Effect of blue and red LED lights on the expression of carotenoid

metabolism related genes in juice sacs of the three citrus varieties. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon. Columns and bars represent the means and SE (n=3), respectively. The letters a, b and c indicate significant differences among the control, blue light and red light treatments at the 5% level by Tukey's HSD test.

Fig. 7. Effect of sucrose and mannitol on the carotenoid content in juice sacs of the three citrus varieties. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon. Total car, total carotenoids. β-car, β-carotene. β-cry, β-cryptoxanthin. T-vio, all-trans-violaxanthin. C-vio, 9-*cis*-violaxanthin. Lut, lutein. The value for total carotenoids was the sum of identified carotenoids. Columns and bars represent the means and SE (n=3), respectively. The letters a, b and c indicate significant differences among the control, sucrose and mannitol treatments at the 5% level by Tukey's HSD test.

Fig. 8. Effect of sucrose and mannitol on the expression of carotenoid metabolism related genes in juice sacs of the three citrus varieties. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon. Columns and bars represent the means and SE (n=3), respectively. The letters a, b and c indicate significant differences among the control, sucrose and mannitol treatments at the 5% level by Tukey's HSD test.

Fig. 9. Effect of ABA and GA on the carotenoid content in juice sacs of the three citrus varieties. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon. Total car, total carotenoids. β-car, β-carotene. β-cry, β-cryptoxanthin. T-vio,

all-trans-violaxanthin. C-vio, 9-*cis*-violaxanthin. Lut, lutein. The value for total carotenoids was the sum of identified carotenoids. Columns and bars represent the means and SE (n=3), respectively. The letters a, b and c indicate significant differences among the control, ABA and GA treatments at the 5% level by Tukey's HSD test.

Fig. 10. Effect of ABA and GA on the expression of carotenoid metabolism related genes in juice sacs of the three citrus varieties. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon. Columns and bars represent the means and SE (n=3), respectively. The letters a, b and c indicate significant differences among the control, ABA and GA treatments at the 5% level by Tukey's HSD test.

Fig. 1

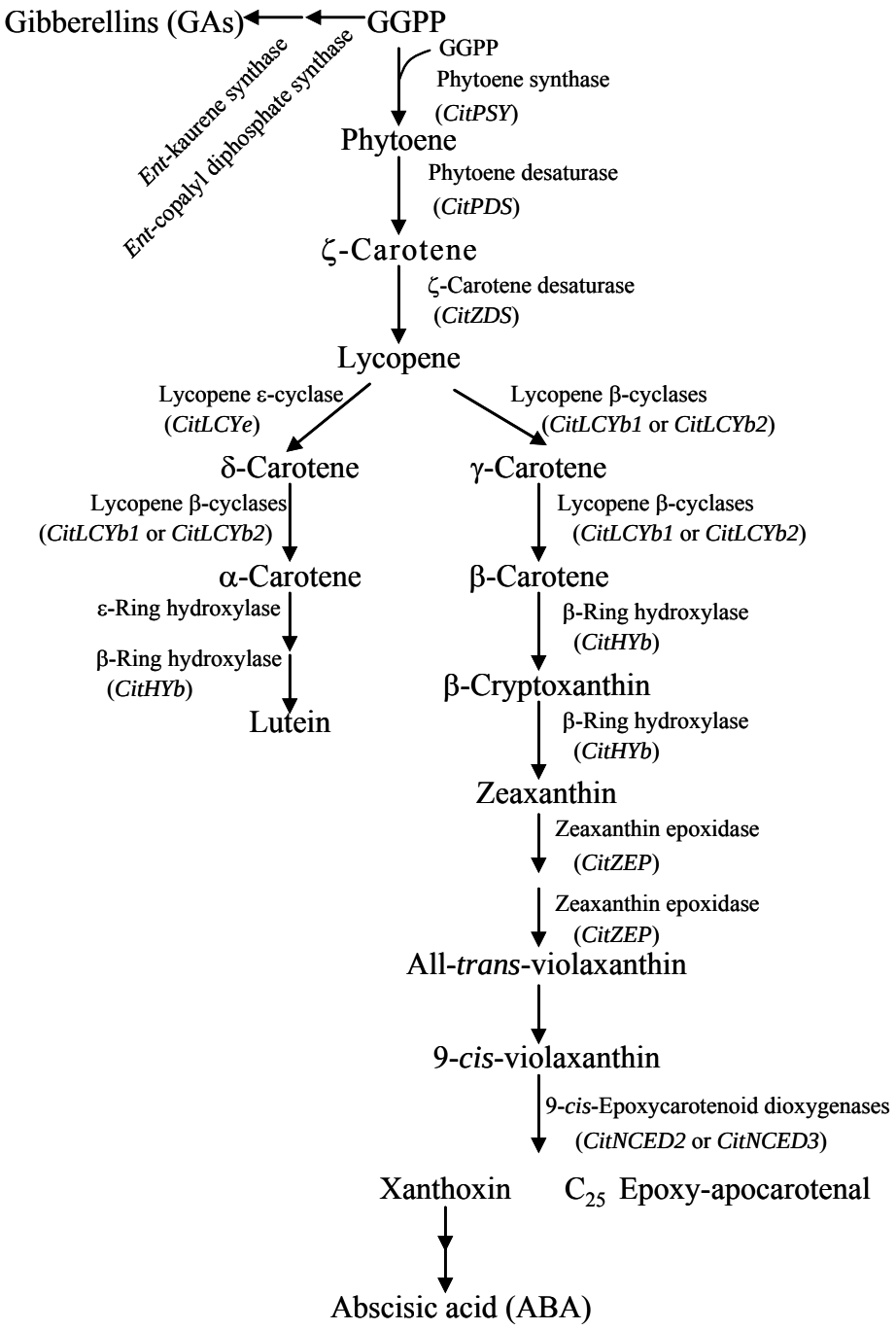

Gibberellins (GAs)
GGPP
Ent-kaurene synthase
Ent-copalyl diphosphate synthase
GGPP
Phytoene synthase
(CitPSY)
Phytoene
Phytoene desaturase
(CitPDS)
ζ-Carotene
ζ-Carotene desaturase
(CitZDS)
Lycopene
Lycopene ε-cyclase
(CitLCYe)
Lycopene β-cyclases
(CitLCYb1 or CitLCYb2)
δ-Carotene
γ-Carotene
Lycopene β-cyclases
(CitLCYb1 or CitLCYb2)
Lycopene β-cyclases
(CitLCYb1 or CitLCYb2)
α-Carotene
β-Carotene
ε-Ring hydroxylase
β-Ring hydroxylase
(CitHYb)
β-Ring hydroxylase
(CitHYb)
Lutein
β-Cryptoxanthin
β-Ring hydroxylase
(CitHYb)
Zeaxanthin
Zeaxanthin epoxidase
(CitZEP)
Zeaxanthin epoxidase
(CitZEP)
All-trans-violaxanthin
9-cis-violaxanthin
9-cis-Epoxycarotenoid dioxygenases
(CitNCED2 or CitNCED3)
Xanthoxin
C25 Epoxy-apocarotenal
Abscisic acid (ABA)

Fig. 2

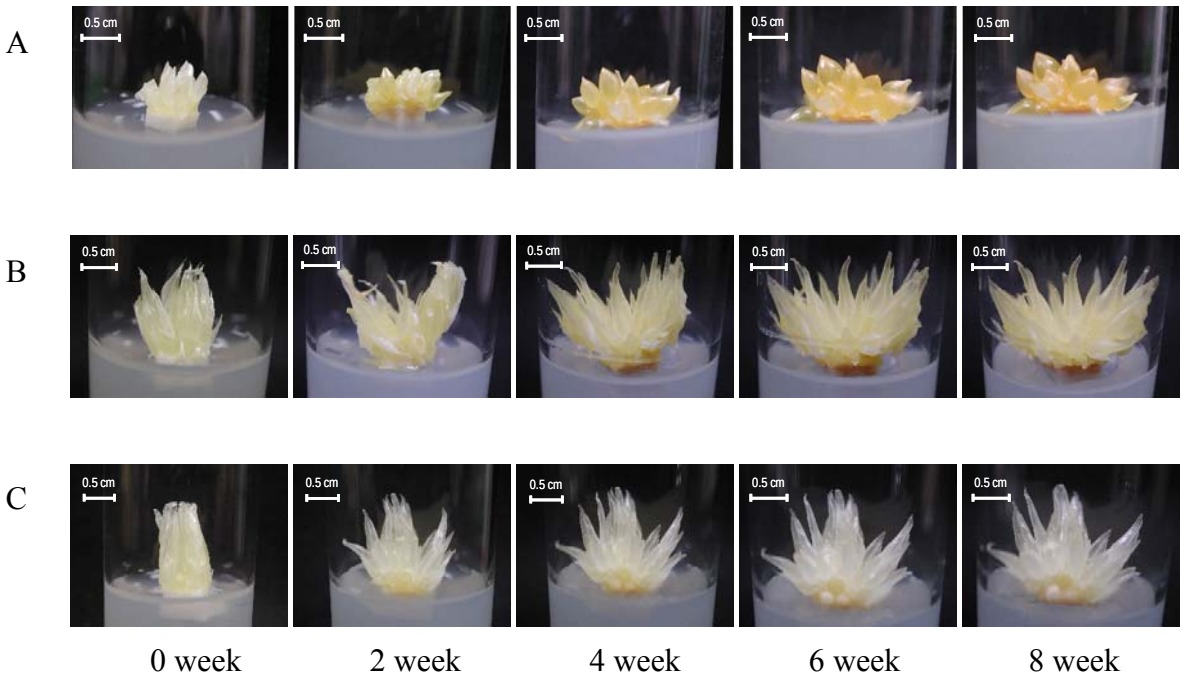


Fig. 3

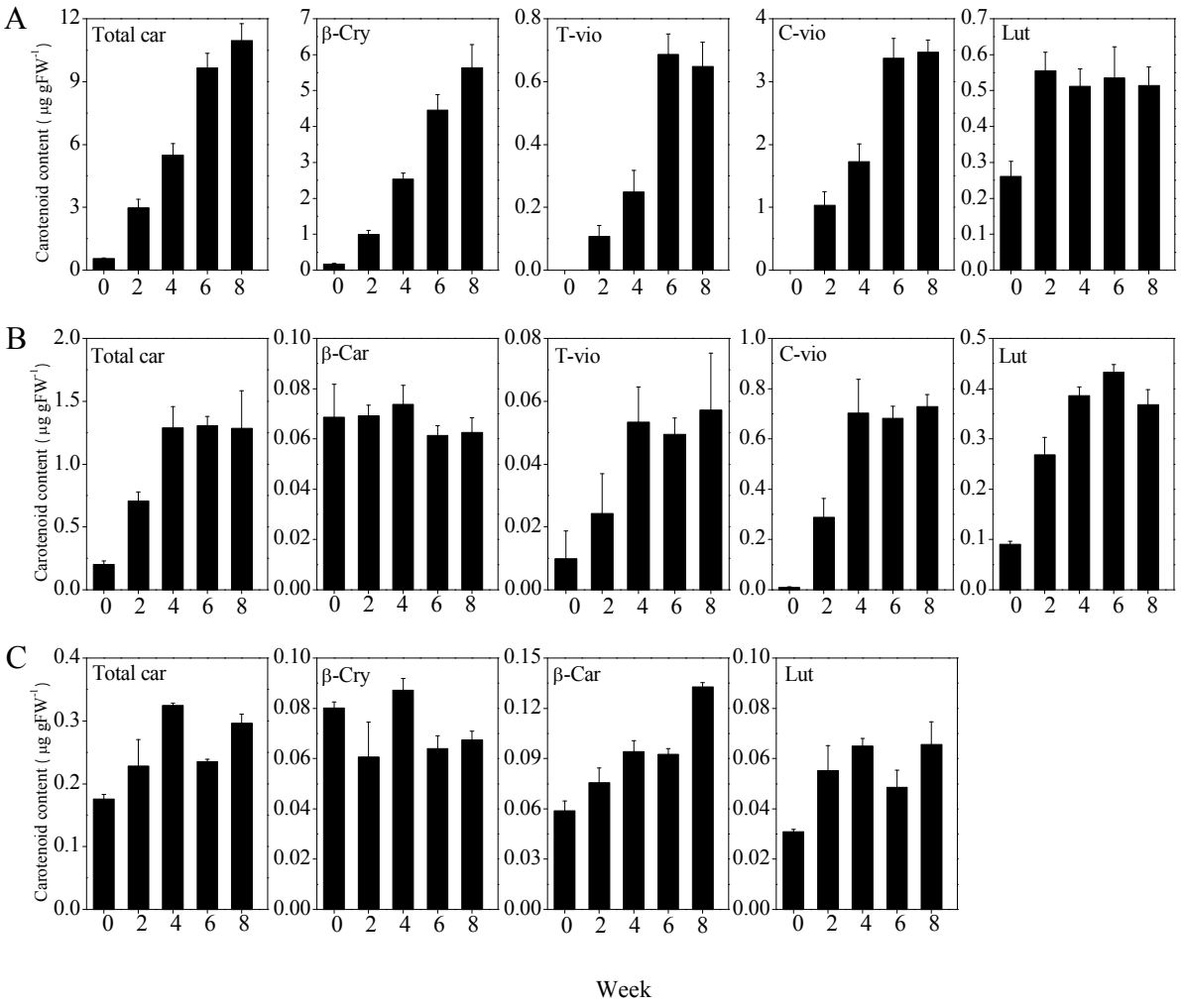

A
Total car
β-Cry
T-vio
C-vio
Lut
B
Total car
β-Car
T-vio
C-vio
Lut
C
Total car
β-Cry
β-Car
Lut
Carotenoid content (μg gFW-1)
Week

Fig. 4

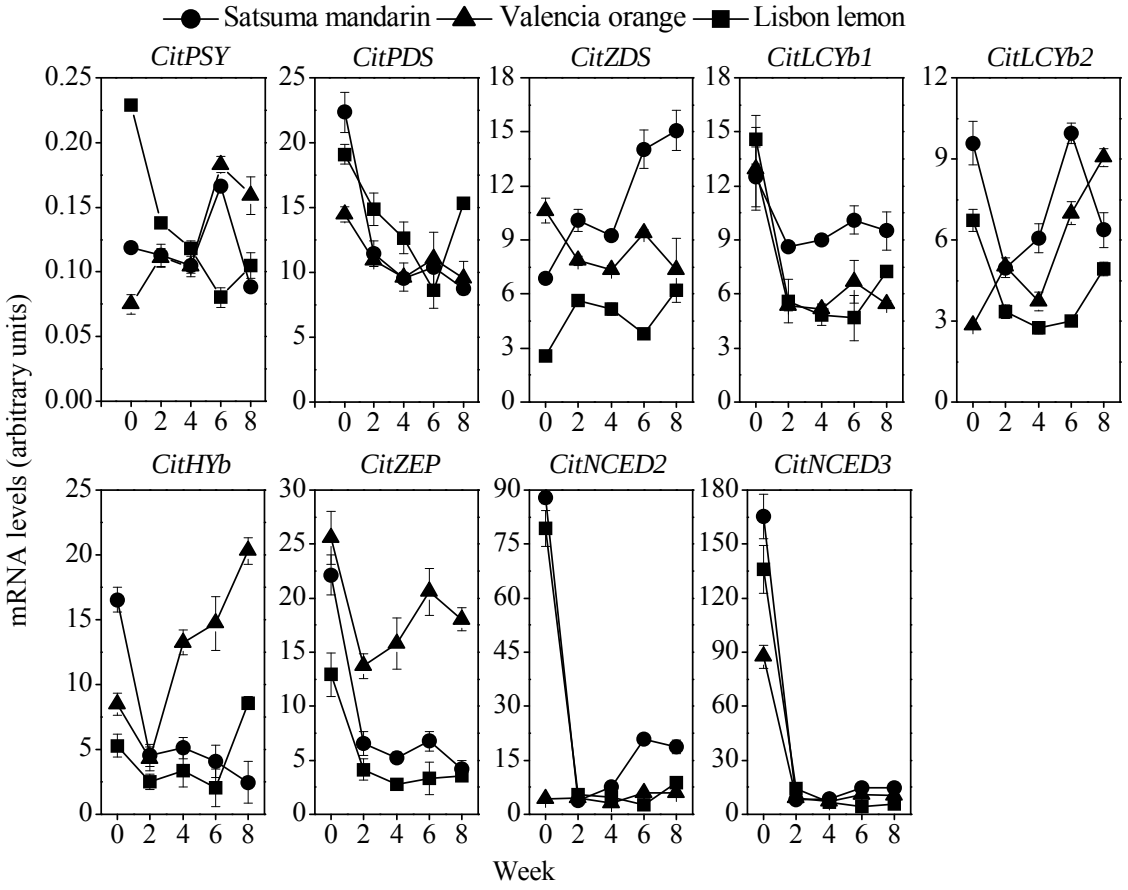

Satsuma mandarin
Valencia orange
Lisbon lemon
CitPSY
CitPDS
CitZDS
CitLCYb1
CitLCYb2
CitHYb
CitZEP
CitNCED2
CitNCED3
mRNA levels (arbitrary units)
Week

Fig. 5

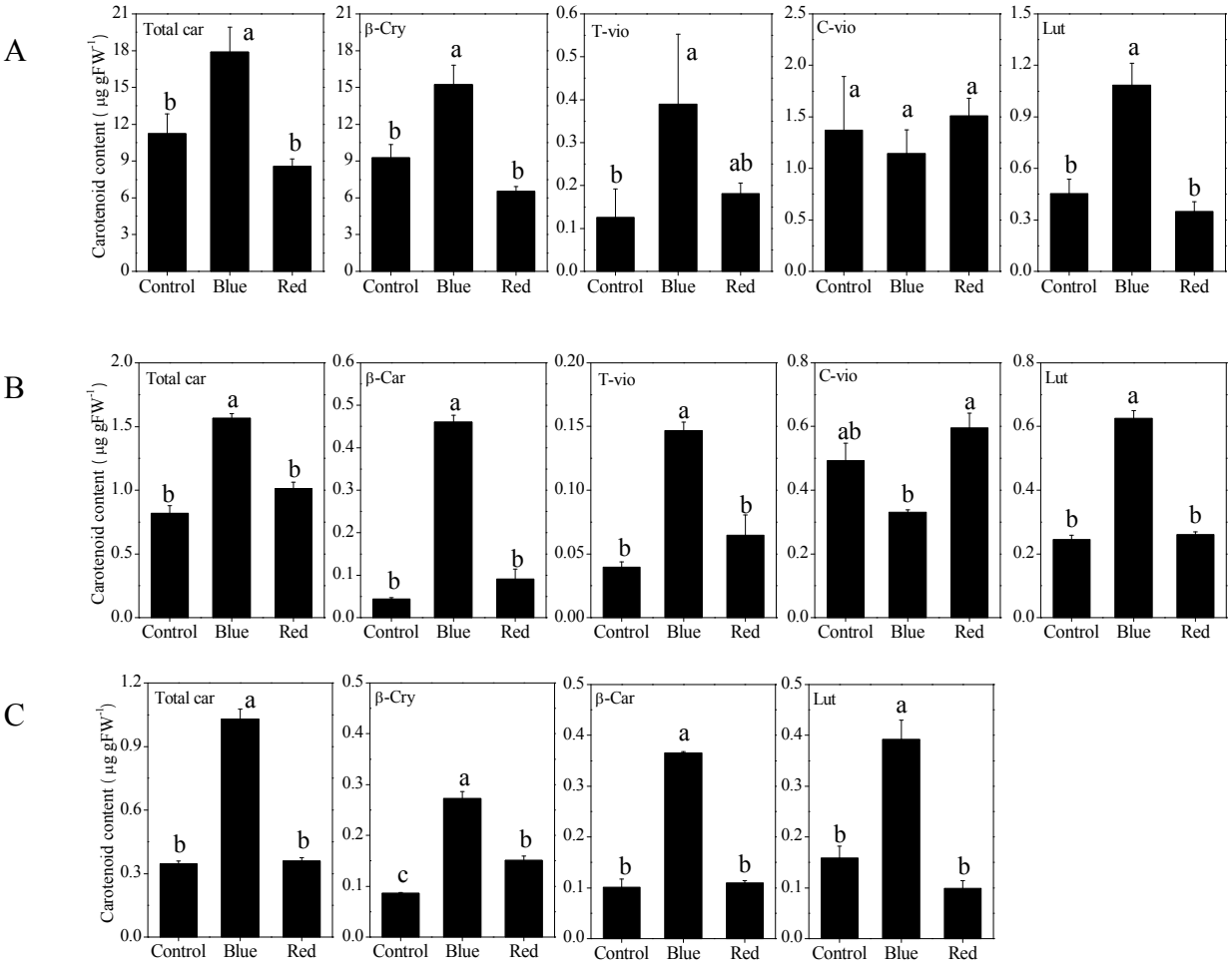

A
Total car
β-Cry
T-vio
C-vio
Lut
Carotenoid content (μg gFW^{-1})
Control
Blue
Red
B
Total car
β-Car
T-vio
C-vio
Lut
Carotenoid content (μg gFW^{-1})
Control
Blue
Red
C
Total car
β-Cry
β-Car
Lut
Carotenoid content (μg gFW^{-1})
Control
Blue
Red

Fig. 6

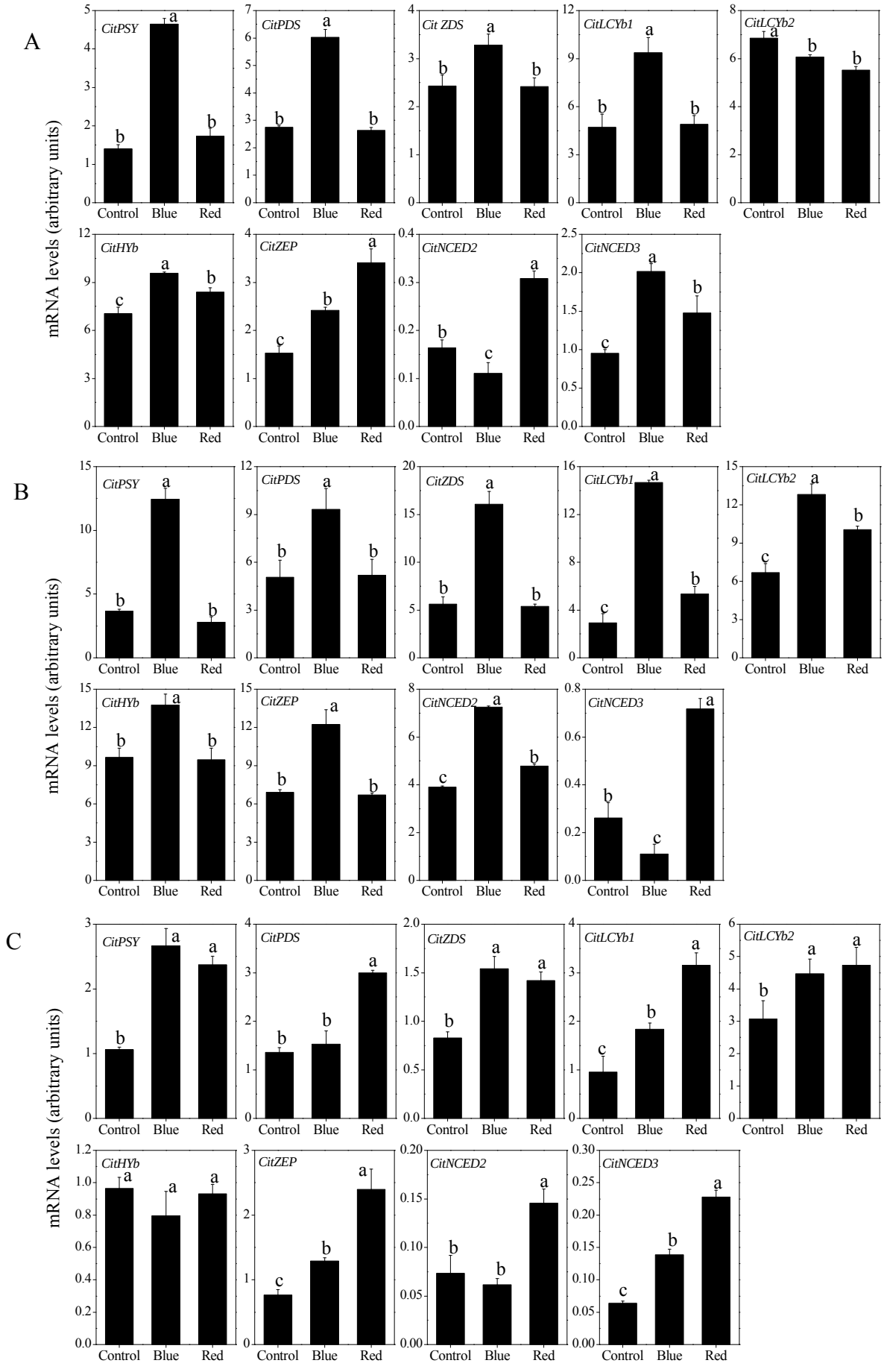

A
B
C
mRNA levels (arbitrary units)
CitPSY
CitPDS
Cit ZDS
CitLCYb1
CitLCYb2
CitHYb
CitZEP
CitNCED2
CitNCED3
CitZDS
Control
Blue
Red

Fig. 7

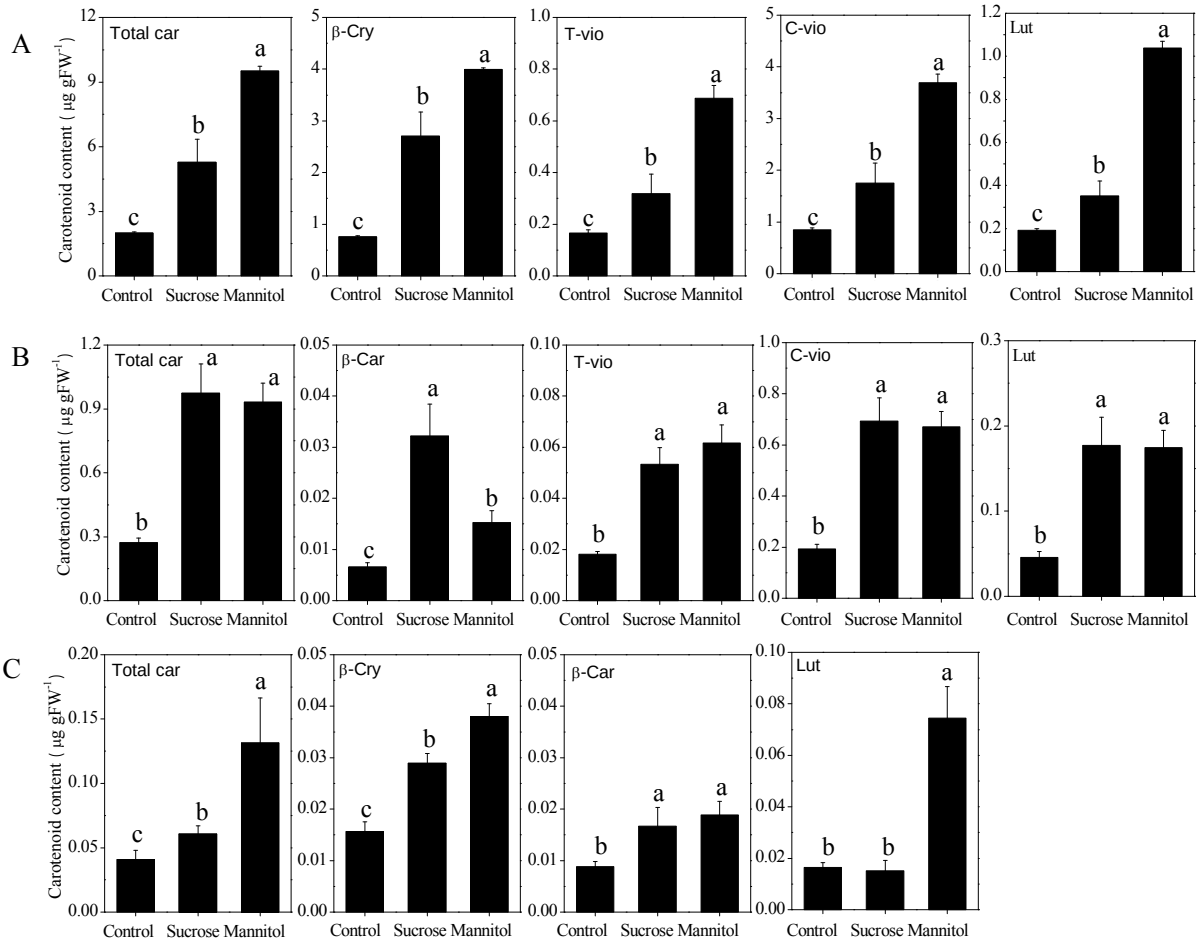

A
B
C
Total car
β-Cry
T-vio
C-vio
Lut
β-Car
Carotenoid content (μg gFW^{-1})
Control
Sucrose
Mannitol

Fig. 8

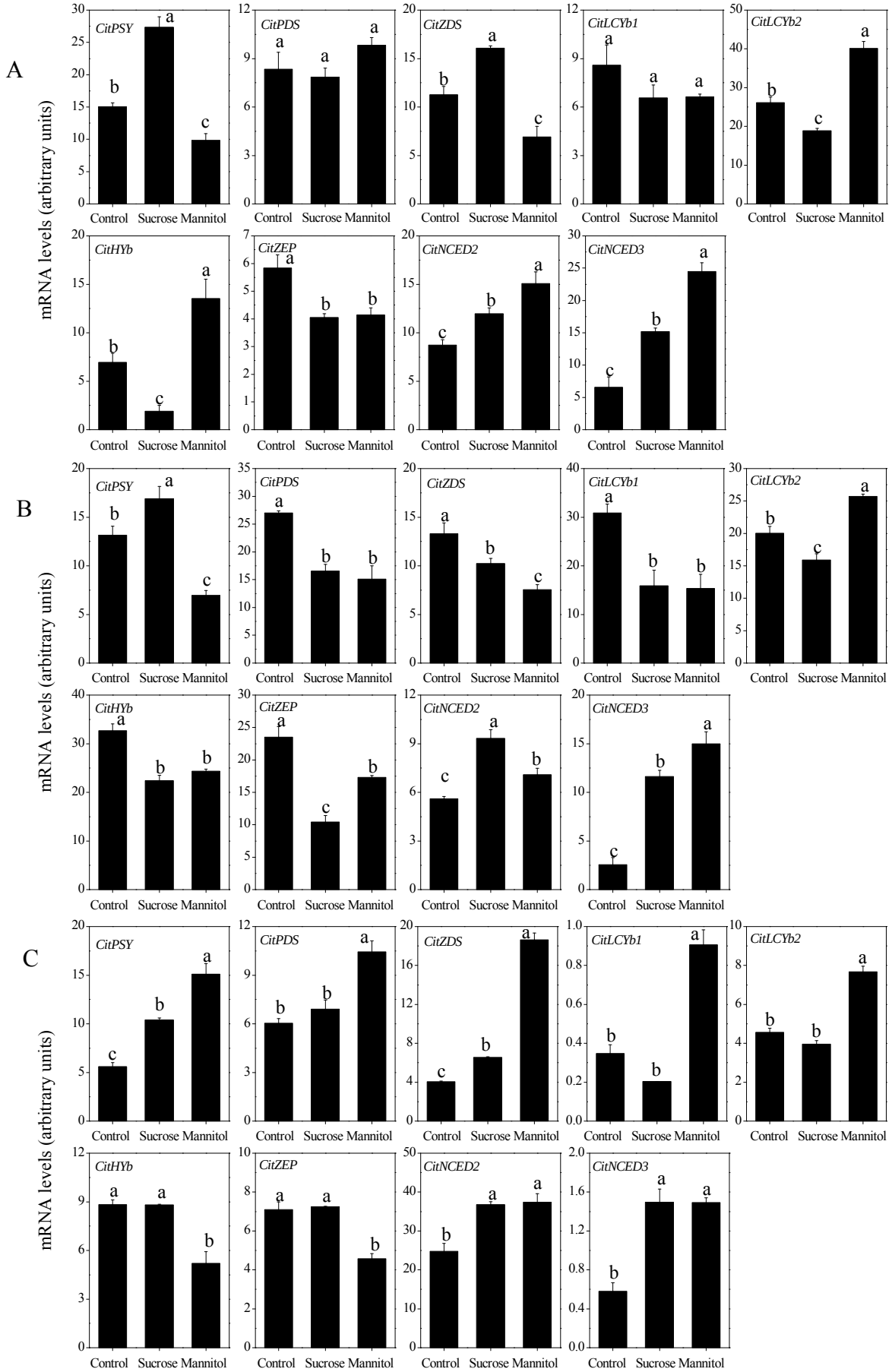

A
B
C
mRNA levels (arbitrary units)
CitPSY
CitPDS
CitZDS
CitLCYb1
CitLCYb2
CitHYb
CitZEP
CitNCED2
CitNCED3
Control
Sucrose
Mannitol

Fig. 9

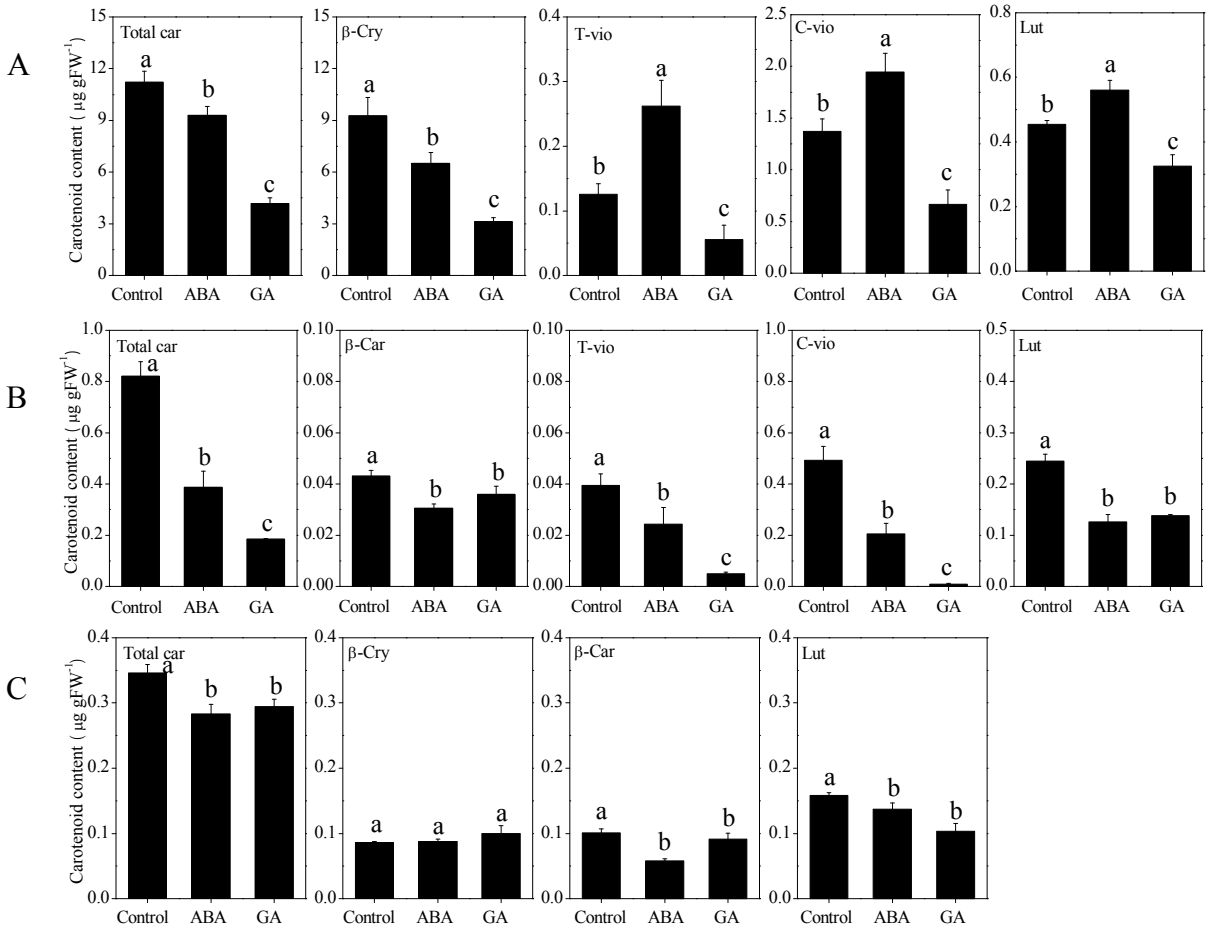
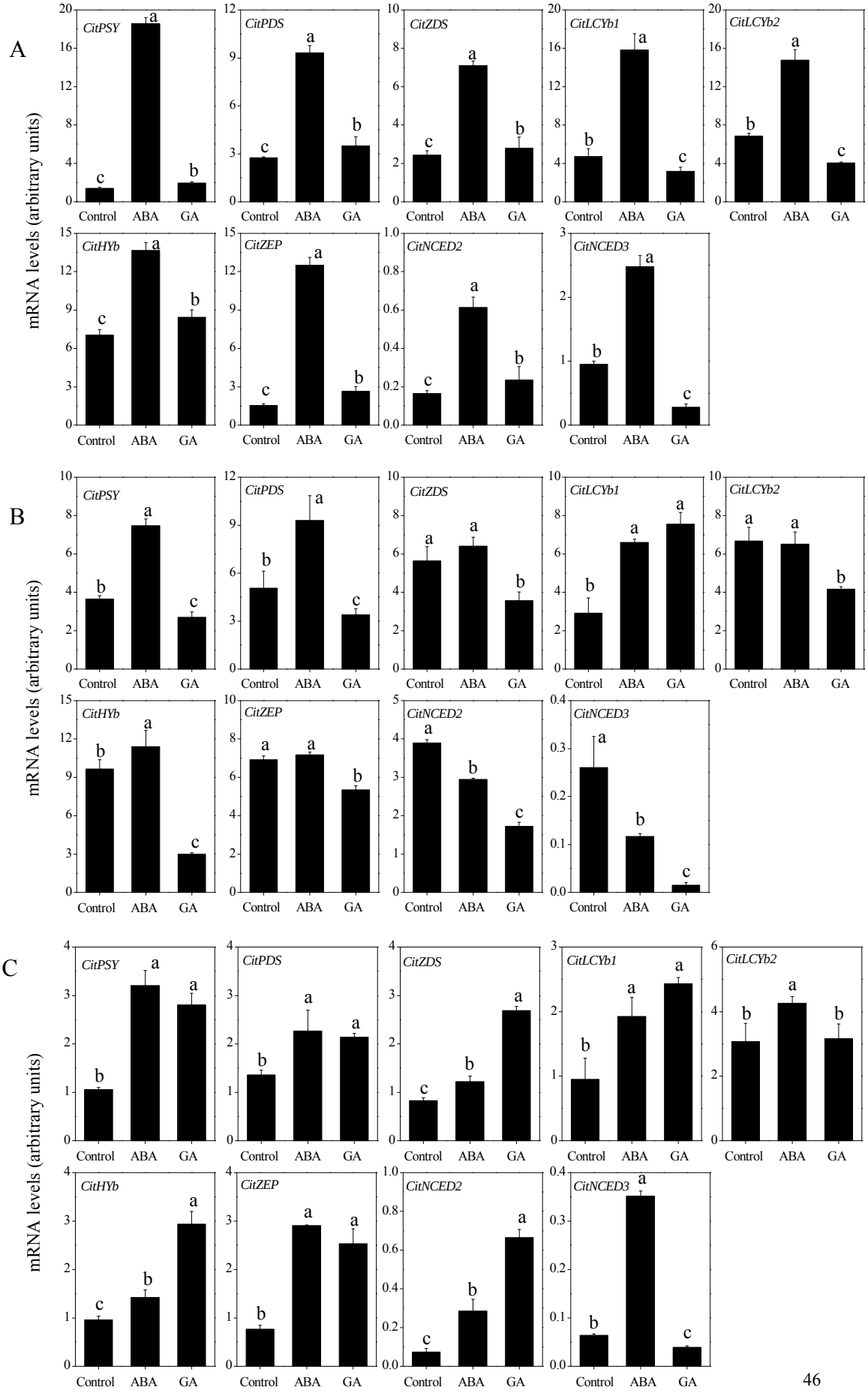

A
B
C
Carotenoid content (μg gFW-1)
Total car
β-Cry
T-vio
C-vio
Lut
β-Car
Control
ABA
GA

Fig. 10


A
B
C
mRNA levels (arbitrary units)
CitPSY
CitPDS
CitZDS
CitLCYb1
CitLCYb2
CitHYb
CitZEP
CitNCED2
CitNCED3
Control
ABA
GA

Regulation of carotenoid accumulation and the expression of carotenoid metabolic genes in citrus juice sacs *in vitro*

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Supplemental Table 1 The changes in ascorbic acid content in the juice sacs cultured *in vitro* and *in vivo*

	Ascorbic acid content (μmol g^{-1})					
	Satsuma mandarin		Valencia orange		Lisbon lemon	
	0 week	8 week	0 week	8 week	0 week	8 week
In vitro	2.16± 0.39	1.86±0.14	5.85± 0.07	2.79±0.21	6.26± 0.21	2.91±0.29
In vivo	2.16± 0.39	1.81±0.23	5.85± 0.07	3.53±0.29	6.26± 0.21	3.71±0.40