Effect of the combination of ethylene and red LED light irradiation on carotenoid accumulation and carotenogenic gene expression in the flavedo of citrus fruit

Running title: Effect of ethylene and red LED lights on carotenoid metabolism in citrus

Effect of the combination of ethylene and red LED light irradiation on carotenoid accumulation and carotenogenic gene expression in the flavedo of citrus fruit

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ABSTRACT

In the present study, the effects of ethylene and red light-emitting diode (LED) light (660 nm) on the accumulation of carotenoids and expression of genes related to carotenoid biosynthesis were investigated in the flavedo of Satsuma mandarin. The results showed that the contents of β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin and lutein were simultaneously increased along with the total carotenoid accumulation by the red LED light. With the ethylene treatment, the contents of β-carotene and β-cryptoxanthin were increased, while the content of lutein was decreased in the flavedo of Satsuma mandarin. The suppression of lutein accumulation by ethylene was inhibited when the ethylene treatment was performed under the red LED light. With the combination of ethylene and red LED light treatments, the contents of β-cryptoxanthin and lutein were simultaneously increased. Gene expression results showed that simultaneous increases in the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitCRTISO*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, and *CitZEP* contributed to the accumulation of β-cryptoxanthin and lutein in the treatment of ethylene combined with red LED light. The results presented herein might provide new strategies to enhance the commercial and nutritional value of citrus fruit.

KEYWORDS: Citrus; β-cryptoxanthin; flavedo; lutein; red LED light

1 Introduction

Carotenoids, important natural isoprenoid pigments, fulfill a variety of important functions in plants and play a critical role in human nutrition and health (Schwartz et al., 1997; Cunningham and Gantt, 1998; Havaux, 1998; Krinsky et al., 2003; Ledford and Niyogi, 2005). In citrus fruit, carotenoids are responsible for the external and internal coloration, and their contents and compositions are important indexes for the commercial and nutritional quality of the fruit. The accumulation of carotenoids in citrus fruit has been extensively investigated over the past decade (Kato et al., 2004; Rodrigo et al., 2004; Kato et al., 2006; Rodrigo and Zacarías, 2007; Kato, 2012; Zhang et al., 2012a; Ma et al., 2013). Moreover, genes encoding enzymes for the main steps of carotenoid biosynthesis have been isolated and their expression was characterized in the flavedo and juice sacs of different citrus varieties (Kato et al., 2004; 2007; Alquézar et al., 2008; Alquézar et al., 2009; Fig. 1). In the 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 accumulation in the flavedo and juice sacs of Satsuma mandarin and Valencia orange (Kato et al., 2004). In addition, the cyclization of lycopene by *CitLCYb1* and *CitLCYb2* played an important role in determining the profiles of carotenoids in the orange stage of the citrus fruit (Zhang et al., 2012b)

Light is an important environmental factor for plants. It is not only an essential energy source for plants, but also an important signal for plants growth and development (Chory et al., 1996; Clouse, 2001; Kim et al., 2002). In higher plants,

sensing of light is carried out by various light photoreceptors (Briggs et al., 2001). Thus, plants exhibit different responses to different wavelengths of lights (Goins et al., 1997; Xu et al., 2011; Jung et al., 2013). Wu et al. (2007) reported that β-carotene content was much higher in the red light-treated group than blue light-treated group in leaves and stems of pea seedlings. In tomatoes, the accumulation of lycopene along with an increase in total carotenoid content was also observed in response to red light treatment (Alba et al., 2000; Schofield and Paliyath, 2005; Liu et al., 2009). In citrus fruit, the red LED light was effective to enhance carotenoids contents, especially the content of β-cryptoxanthin, while blue LED light had no significant effect on the carotenoids contents in the flavedo of Satsuma mandarin (Ma et al., 2012). Even though citrus fruit are non-climacteric and produce a low level of ethylene during ripening, they are sensitive to exogenous ethylene. In the recent year, exogenous application of ethylene has been widely employed to enhance the external coloration of citrus fruit. Rodrigo and Zacarías (2007) reported that exogenous ethylene treatment increased the contents of carotenoids; as a result, the degreening process of citrus fruit was accelerated. To date, however, information on the effects of the combination of ethylene and the red LED light on carotenoid accumulation in citrus is still unknown. As citrus fruit pulp matures earlier than the peel, the pulp reaches maturity and is edible, while the peel is still green in October. In the present study, to promote peel degreening and improve the carotenoids contents and compositions in the flavedo of citrus fruit, the effects of red LED light (660nm) and ethylene on carotenoid accumulation and the expression of genes related to carotenoid

biosynthesis were investigated. The results presented herein might provide new strategies to enhance the carotenoid production in citrus fruit.

2 Materials and methods

2.1 Plant Materials

Fruit of Satsuma mandarin (*Citrus unshiu Marc.)* were harvested in October 150 day after anthesis at the Fujieda Farm of Shizuoka University (Shizuoka, Japan). In this stage, the fruit peel just begins to degreen with the accumulation of carotenoids. Fruit 45-50 mm in diameter and light green in color were used as materials.

2.2 Treatment

Fruit were placed in 35-L sealed plastic chambers. The fruit were continuously treated for 6 days at 20 °C as follows: with 150 μmol $m^{-2}s^{-1}$ red (660 nm) LED lights; with 50 μL L^{-1} ethylene in the dark; with 50 μL L^{-1} of ethylene under 150 μmol $m^{-2}s^{-1}$ red LED lights. Fruit stored at 20 °C in the dark were used as the control. After each treatment, the flavedo was immediately frozen in liquid nitrogen, and kept at – 80 °C until used.

2.3 Color measurement

Color measurement was carried out with a colorimeter (NR-11, Nippon Denshoku, Japan). The CIE 1976 $L^*a^*b^*$ color scale was adopted. The hue angle [H^o=arctangent (b^*/a^*) and Citrus color index (CCI)=[$1000\times a^*/(L^*\times b^*)$] were calculated according to methods previously reported (Zhou et al., 2010).

2.4 Extraction and determination of carotenoids

The identification, extraction and quantification of carotenoid in citrus have been

described previously (Kato et al., 2004). β-Carotene, β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin and lutein were quantified in the flavedo of Satsuma mandarin during the experimental period. The contents of carotenoids were expressed as μg g^{-1} fresh weight. Carotenoid quantification was performed in three replicates.

2.4 Total RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from the flavedo of Satsuma mandarin fruit according to a previously reported method (Kato et al., 2004). 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*, *CitCRTISO*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, *CitZEP* and *CitVDE* were designed according to Ma et al. (2013; Table 1). 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 StepOnePlus™ Real-Time PCR System (Applied Biosystems) according to the manufacture's instructions. Each reaction mixture 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

StepOnePlus™ Real-Time PCR 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.

2.5 Statistical analysis

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

3 Results and discussion

3.1 Effects of red LED light and ethylene on fruit color

In the present study, the changes in the color were described by the hue angle (H^o) and CCI. H^o value ranges from 0^o to 360^o with 0^o for red-purple, 90^o for yellow, and 180^o for bluish green. For CCI, positive values are for red, negative values are for blue-green, and 0 is for an intermediate mixture of red, yellow, and blue-green. In the control, the H^o decreased while the CCI increased during the storage, indicating that the color of flavedo turned yellow gradually. Both ethylene and red LED light treatments can accelerate the changes in H^o and CCI in flavedo of Satsuma mandarin during the storage. In the combination of ethylene and red LED light treatments, the changes in H^o and CCI were more significant than ethylene or red LED light treatment alone, and the color of flavedo turned deeper yellow color on the sixth day (Fig. 2 A and B). In addition, as shown in the Fig. 2 C, the peel turned completely yellow in the ethylene treatment on the sixth day after harvest. In the treatment of red LED light, non-uniform color with light green were observed in the peel on the sixth

149 day after harvest. These result suggested that ethylene is more effective to induce
150 chlorophyll breakdown than the red LED irradiation, which led to a better color in the
151 ethylene treatment on the six day after harvest.

152 *3.2 Effects of red LED light and ethylene on carotenoid content and composition*

153 We previously reported that irradiation with red light at intensity of 50 μmol $m^{-2}s^{-1}$
154 for six days was effective to enhance carotenoids contents, especially the content of
155 β-cryptoxanthin; while blue LED light had no significant effect on the carotenoid
156 content in the flavedo of Satsuma mandarin (Ma et al., 2012). In the present study, we
157 increased the intensity of the red LED light to 150 μmol $m^{-2}s^{-1}$, and the results showed
158 that the contents of β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin and
159 lutein were simultaneously increased along with the total carotenoid accumulation
160 under red LED light. With the ethylene treatment, the contents of β-carotene and
161 β-cryptoxanthin were increased, while the contents of lutein, all-*trans*-violaxanthin,
162 and 9-*cis*-violaxanthin were decreased. The total carotenoid content was not
163 significantly affected by the ethylene treatment. In contrast, when the ethylene
164 treatment was performed under red LED light, the contents of β-carotene,
165 β-cryptoxanthin, all-*trans*-violaxanthin and lutein were increased, and the total
166 carotenoid content was higher than that in the ethylene treatment alone (Fig. 3).

167 In the present study, the results showed that ethylene exhibited different effects on
168 the accumulation of individual carotenoids. The ethylene treatment decreased the
169 content of lutein, which is the major β,ε-carotenoid accumulating in the green stage.
170 While it increased the contents of β-carotene and β-cryptoxanthin in the flavedo of

171 Satsuma mandarin. In citrus fruit, the carotenoid biosynthetic pathway changes from
172 β,ε-carotenoid synthesis to β,β-carotenoid synthesis during the ripening process (Kato
173 et al., 2004; Zhang et al., 2012b). In this study, the changes of the carotenoids
174 compositions in the flavedo of ethylene-treated fruit resembled those ripening on tree.
175 These results suggested that ethylene was involved in the regulation of carotenoid
176 accumulation in citrus fruit during the ripening process. The similar results were also
177 observed in Navelate oranges (Rodrigo and Zacarías, 200). Matsumoto et al. (2009)
178 reported that ethylene treatment increased the contents of carotenes and
179 β-cryptoxanthin, and as a result the total carotenoid content was increased
180 dramatically in the flavedo of Satsuma mandarin harvested in the orange stage.
181 However, the results presented herein showed that the ethylene treatment did not
182 increase the total carotenoid content in the flavedo of Satsuma mandarin harvested in
183 the green stage because of the rapid decrease of lutein. Thus, the effects of ethylene
184 on the total carotenoid accumulation in citrus fruit were distinct in the different
185 ripening stages.

186 In addition, we found that the suppression effect of ethylene on lutein accumulation
187 in the flavedo of the green stage could be inhibited by red LED light irradiation. The
188 contents of lutein, β-carotene, β-cryptoxanthin, and all-*trans*-violaxanthin were
189 simultaneously increased when the ethylene treatment was performed under red LED
190 light. In general, consumers eat the pulp of citrus fruits. In the recent years, however,
191 citrus peel becomes more popular in human diet, for example it is added fresh as zest,
192 or dried and candied for use in food and drinks. It has been proven that consuming the

citrus peel is beneficial to human health because of its high levels of vitamins, minerals, antioxidants and carotenoids. In Satsuma mandarin, the contents of carotenoids and antioxidants are much higher in the peel than the pulp (Kato et al., 2004). In the present study, the results showed that the contents of β-cryptoxanthin and lutein, which are important antioxidants beneficial to human health, were increased by the combination treatments of ethylene and red LED light. These results suggested that the postharvest application of ethylene combined with the red LED light irradiation contributed to improve the nutritional value of citrus fruit.

3.2 Effects of red LED light and ethylene on gene expression related to carotenoid metabolism

The transcriptional regulation of carotenoid biosynthetic genes is a major mechanism by which the accumulations of specific carotenoids are regulated in the flavedo and juice sacs (Kato et al., 2004, 2006; Ma et al., 2012; Zhang et al., 2012). As shown in Fig. 4, the expression of *CitPSY*, *CitCRTISO*, *CitLCYb2*, *CitLCYe*, and *CitVDE* was up-regulated by the red LED light treatment. With the ethylene treatment, the expression of a set of genes related to β,β-xanthophylls biosynthesis (*CitPSY*, *CitPDS*, *CitZDS*, *CitCRTISO*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, and *CitZEP*) was simultaneously up-regulated. In contrast, the expression of *CitLCYe* was down-regulated by ethylene. Under red LED light, the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitCRTISO*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, and *CitZEP* was significantly up-regulated by the ethylene treatment, which contributed to the accumulation of β-cryptoxanthin and lutein. Moreover, the expression levels of

CitPSY, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, and *CitHYb* were higher than those treated by ethylene alone.

In citrus fruit, the drastically decrease in the expression of *CitLCYe* was responsible for the decrease of lutein, and accelerated the pathway from β,ε-carotenoid synthesis to β,β-carotenoid synthesis during the ripening process (Kato et al., 2004; Zhang et al., 2012b). In the present study, the results showed that the ethylene treatment down-regulated expression of *CitLCYe*, which led to the decrease of lutein. However, when the ethylene treatment was performed under red LED light, the expression of *CitLCYe* was significantly increased, and the simultaneous increases in the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, and *CitZEP* contributed to the accumulation of β-cryptoxanthin and lutein in the ethylene treatment under the red LED light.

4. Conclusion

In the present study, the effects of combination of red LED light and ethylene on carotenoid accumulation in the flavedo of citrus fruit have been studied. The results showed that the combination of ethylene treatment and the red LED light was effective to increase the contents of β-cryptoxanthin and lutein in the flavedo of citrus fruit. Moreover, simultaneous increases in the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitCRTISO*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, and *CitZEP* contributed to the accumulation of β-cryptoxanthin and lutein in the ethylene treatment under the red LED light. These results indicated that the combination of ethylene and red LED light treatment was effective to enhance the commercial and nutritional value of citrus fruit.

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FIGURE LEGENDS

Fig. 1. Carotenoid biosynthetic pathway in citrus. GGPP, geranylgeranyl diphosphate. Enzymes are named according to the designation of their genes. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; HYe, ε-ring hydroxylase; HYb, β-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase.

Fig. 2. Effect of red LED light and ethylene on fruit color during the storage. A, H^o. B, CCI. C, The photoes of the citrus fruit on the sixth day after harvest. H^o=arctangent (b^*/a^*) ; Citrus color index (CCI)=[$1000\times a^*/(L^*\times b^*)$] .

Fig. 3. Effect of red LED light and ethylene on the carotenoid content in the flavedo of citrus fruit. β-Car, β-carotene. β-Cry, β-cryptoxanthin. T-vio, all-trans-violaxanthin. C-vio, 9-*cis*-violaxanthin. Lut, lutein. Total car, Total carotenoid. C, control. R, red. E, ethylene. R+E, combination treatment with red and ethylene. The value for total carotenoid was the sum of identified carotenoids. Columns and bars represent the means and SE (n=3), respectively. Different letters indicate significant differences at the 5% level by Tukey's HSD test.

Fig. 4. Effect of red LED light and ethylene on the expression of carotenoid metabolism related genes in the flavedo of citrus fruit. C, control. R, red. E, ethylene. R+E, combination treatment with red and ethylene. The mRNA levels were analyzed by TaqMan real-time quantitative RT-PCR. Real-time RT-PCR amplification of 18S ribosomal RNA was used to normalize the expression of the genes under identical

conditions. Columns and bars represent the means and SE (n=3), respectively. Different letters indicate significant differences at the 5% level by Tukey’s HSD test.

Table 1 Primer sequences and TaqMan MGB Probes used for the quantitative RT-PCRs of the genes related to carotenoid metabolism

cDNA	Primer sequence	TaqMan MGB Probe
CitPSY	Sense: CGTTGATGGGCCTAATGCTT	ACACATAACTCCAACAGC
	Antisense :ACCTGGACTCCCACCTGTCTAA	
CitPDS	Sense: TGGCAACCCCCCAGAGA	ACTTTGCTTGCCTATTGT
	Antisense: CACCCAGTGACTGAATGTGTT	
CitZDS	Sense: AAAGGCACTTGTTGATCCTGATG	CCTTGAAGGACATACGAGAT
	Antisense: ACCAATCAGAGAAGCTTATACTATCCA	
CitCRTISO	Sense: AAAGACACACCGGCGGTATC	AGCTCGCGATCAGG
	Antisense: CGAGGCATTGGCCCATAG	
CitLCYb1	Sense: TGGTACCGCTGGGATGGT	CACCCTTCAACTGGCT
	Antisense: CAATAGGAGCCGCAGCTAAAGT	
CitLCYb2	Sense: CCTTGGCTCAACCAGGATGA	CAGAGGCAGGCCAC
	Antisense: ACCCATTCCACACTTTCTGATGA	
CitLCYe	Sense: AAGGTGTGTCGAGTCAGGTGTTT	ATATCTTAGCTCAAAAGTGG
	Antisense: CCACTGGTAGATTCCGTAATGCT	
CitHYb	Sense: GCGGCTCACCAGCTTCAC	ACTCGGATAAATTCC
	Antisense: CCGAGAAAGAGCCCATATGG	
CitZEP	Sense: CTAAAGAGCTATGAGAGAGCTAGGAGACT	CGAGTGGCTGTTATC
	Antisense: CACTGCGGCCGATCTTG	
CitVDE	Sense: CAAAGACTTCAATGGGAAGTGGTA	TTTCTAGTGGTTTAAATCC
	Antisense: TGGCAATCAAAAGTATCGAAGGA	

Fig. 1

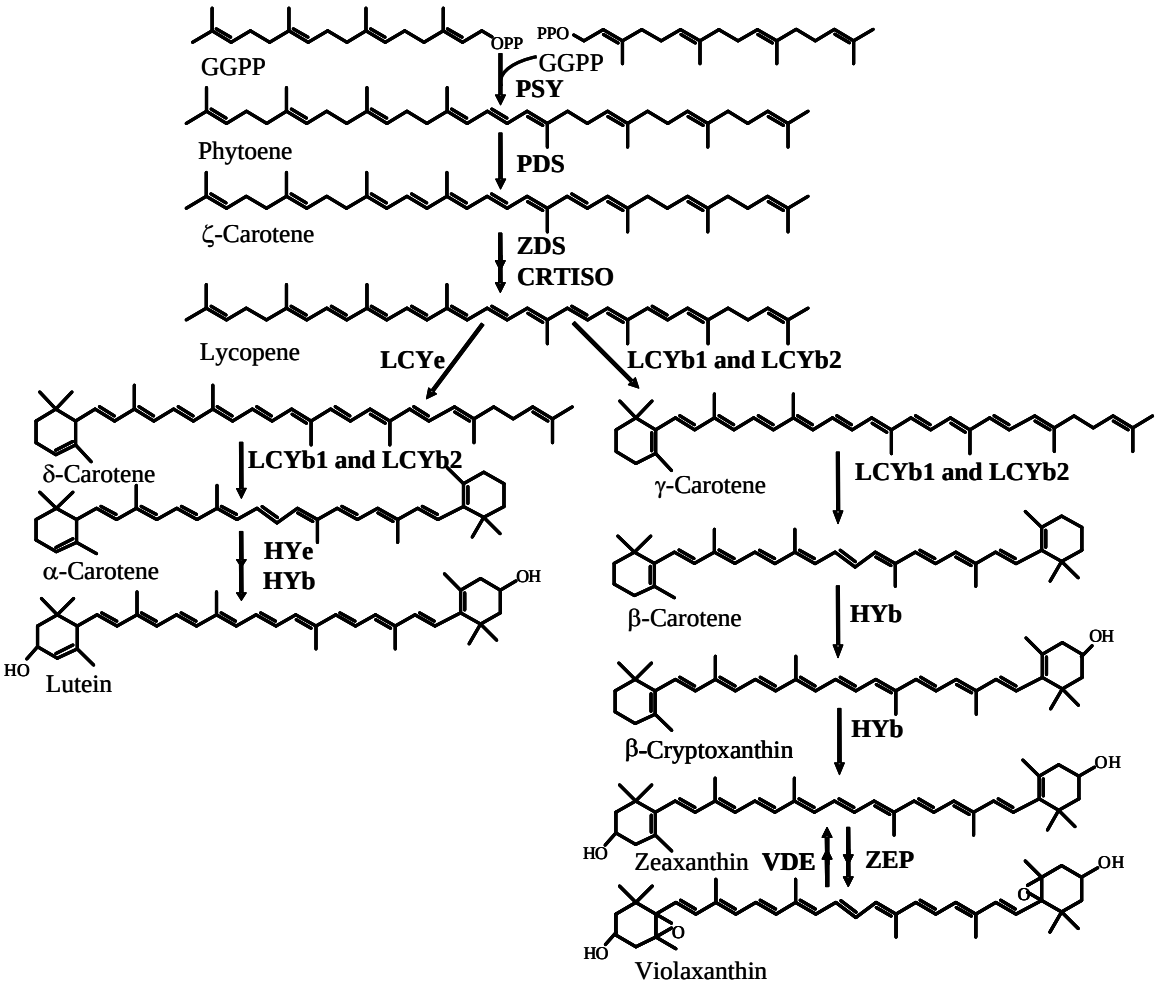

GGPP
OPP
PPO
GGPP
PSY
Phytoene
PDS
ζ-Carotene
ZDS
CRTISO
Lycopene
LCYe
LCYb1 and LCYb2
δ-Carotene
LCYb1 and LCYb2
α-Carotene
HYe
HYb
OH
HO
Lutein
γ-Carotene
LCYb1 and LCYb2
β-Carotene
HYb
OH
β-Cryptoxanthin
HYb
OH
HO
Zeaxanthin
VDE
ZEP
OH
O
HO
Violaxanthin

Fig. 2

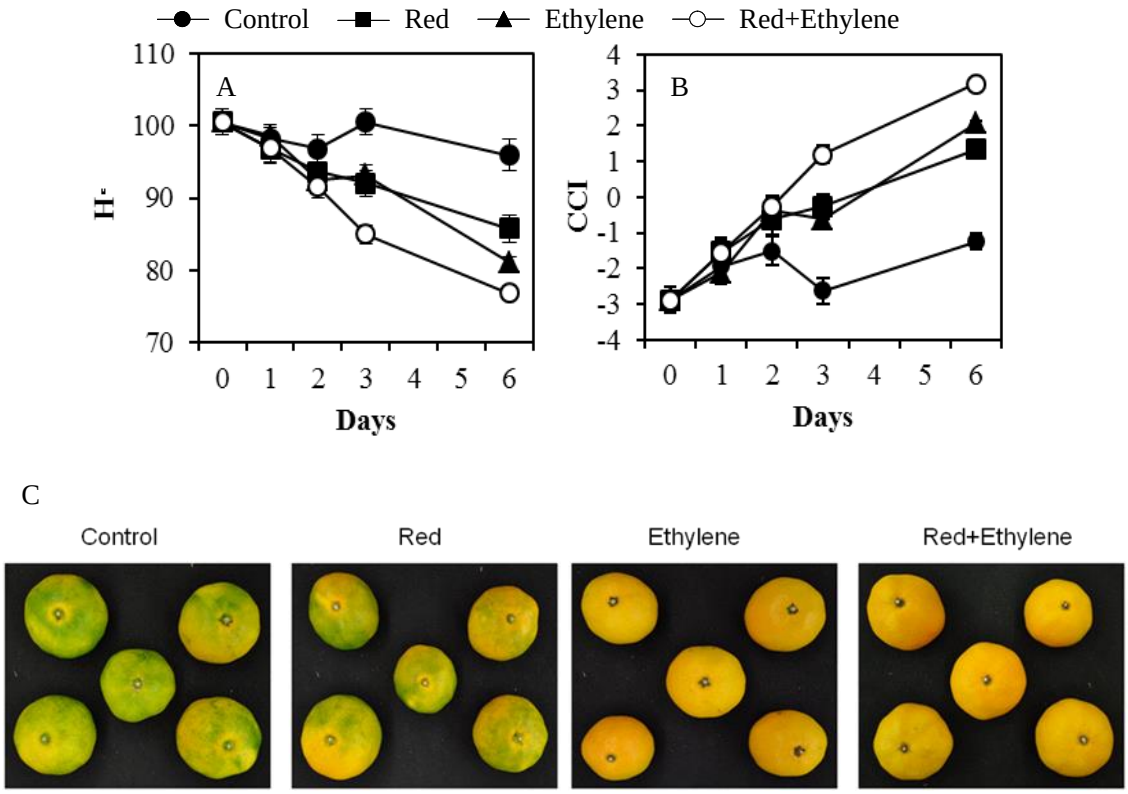

Control
Red
Ethylene
Red+Ethylene
A
B
H°
CCI
Days
Days
C
Control
Red
Ethylene
Red+Ethylene

Fig. 3

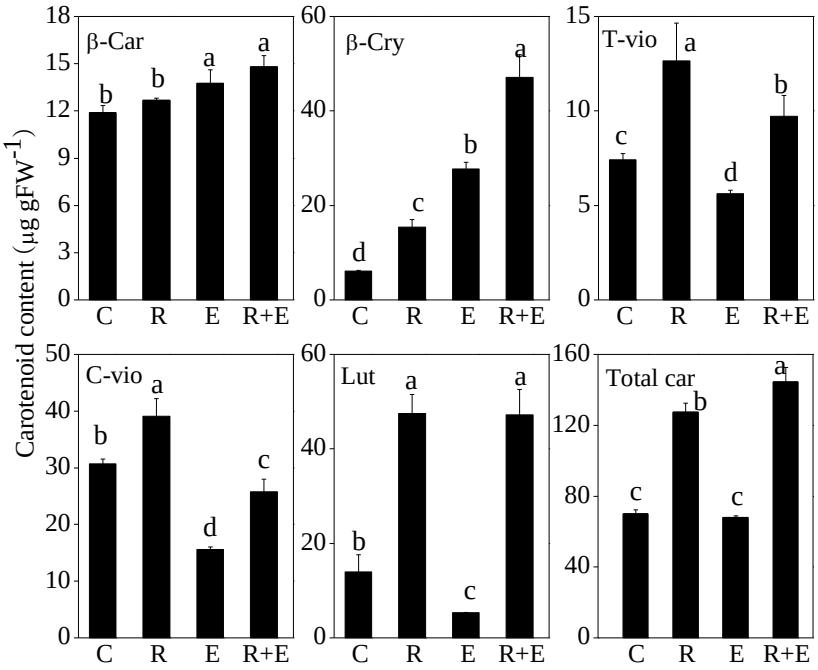
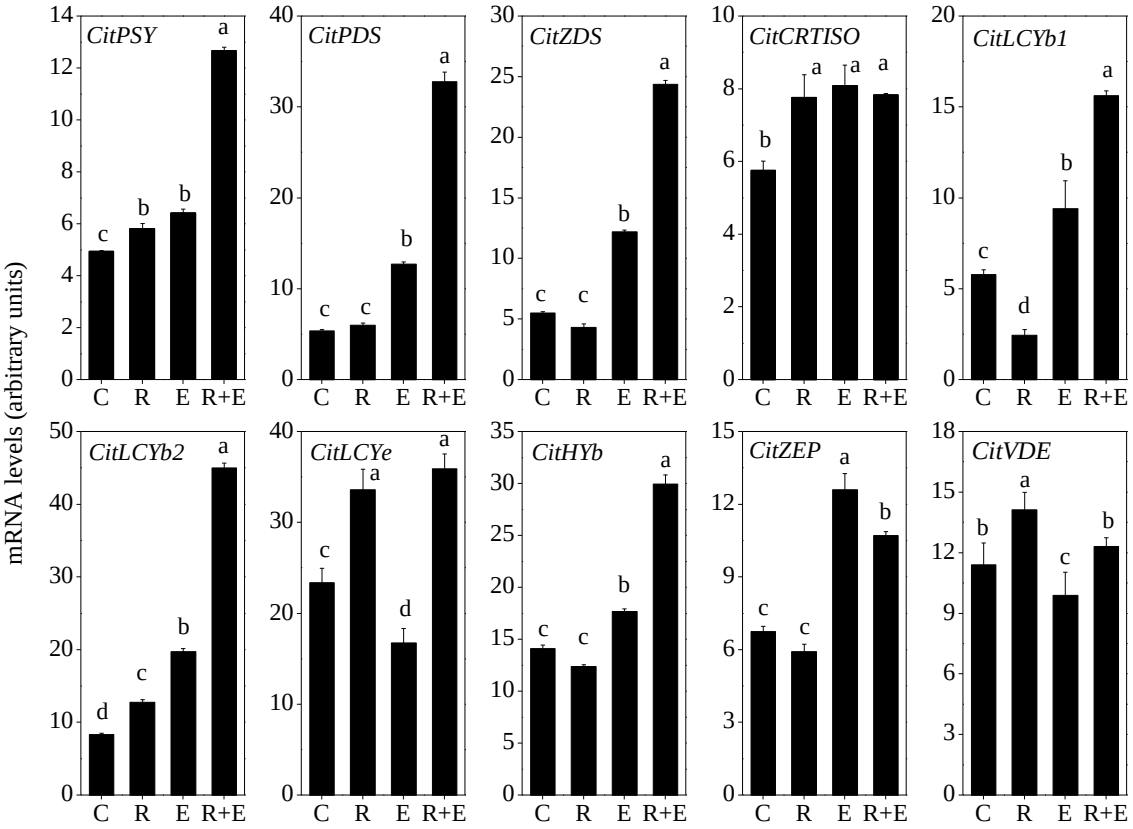

Carotenoid content (μg gFW-1)
β-Car
β-Cry
T-vio
C-vio
Lut
Total car
C
R
E
R+E

Fig. 4


mRNA levels (arbitrary units)
CitPSY
CitPDS
CitZDS
CitCRTISO
CitLCYb1
CitLCYb2
CitLCYe
CitHYb
CitZEP
CitVDE
C R E R+E