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journal or publication title	Postharvest Biology and Technology
volume	99
page range	99-104
year	2015-01
出版者	Elsevier
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URL	http://hdl.handle.net/10297/7917

doi: 10.1016/j.postharvbio.2014.08.002

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*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, and *CitZEP*) led to massive β,β -xanthophyll 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 $\mu\text{mol m}^{-2}\text{s}^{-1}$ red (660 nm) LED lights; with 50 $\mu\text{L L}^{-1}$ ethylene in the dark; with 50 $\mu\text{L L}^{-1}$ of ethylene under 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$ 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 [$H^\circ = \arctangent(b^*/a^*)$] and Citrus color index (CCI) [$\text{CCI} = [1000 \times a^* / (L^* \times b^*)]$] 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 $\mu\text{g g}^{-1}$ 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 $\pm$ 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°) and CCI. H° value ranges from 0° to 360° with 0° for red-purple, 90° for yellow, and 180° 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° 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° and CCI in flavedo of Satsuma mandarin during the storage. In the combination of ethylene and red LED light treatments, the changes in H° 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

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

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

We previously reported that irradiation with red light at intensity of $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ for six days was effective to enhance carotenoids contents, especially the content of β -cryptoxanthin; while blue LED light had no significant effect on the carotenoid content in the flavedo of Satsuma mandarin (Ma et al., 2012). In the present study, we increased the intensity of the red LED light to $150 \mu\text{mol m}^{-2}\text{s}^{-1}$, and 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 under red LED light. With the ethylene treatment, the contents of β -carotene and β -cryptoxanthin were increased, while the contents of lutein, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin were decreased. The total carotenoid content was not significantly affected by the ethylene treatment. In contrast, when the ethylene treatment was performed under red LED light, the contents of β -carotene, β -cryptoxanthin, all-*trans*-violaxanthin and lutein were increased, and the total carotenoid content was higher than that in the ethylene treatment alone (Fig. 3).

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

Satsuma mandarin. In citrus fruit, the carotenoid biosynthetic pathway changes from β,ϵ -carotenoid synthesis to β,β -carotenoid synthesis during the ripening process (Kato et al., 2004; Zhang et al., 2012b). In this study, the changes of the carotenoids compositions in the flavedo of ethylene-treated fruit resembled those ripening on tree. These results suggested that ethylene was involved in the regulation of carotenoid accumulation in citrus fruit during the ripening process. The similar results were also observed in Navelate oranges (Rodrigo and Zacarías, 2000). Matsumoto et al. (2009) reported that ethylene treatment increased the contents of carotenes and β -cryptoxanthin, and as a result the total carotenoid content was increased dramatically in the flavedo of Satsuma mandarin harvested in the orange stage. However, the results presented herein showed that the ethylene treatment did not increase the total carotenoid content in the flavedo of Satsuma mandarin harvested in the green stage because of the rapid decrease of lutein. Thus, the effects of ethylene on the total carotenoid accumulation in citrus fruit were distinct in the different ripening stages.

In addition, we found that the suppression effect of ethylene on lutein accumulation in the flavedo of the green stage could be inhibited by red LED light irradiation. The contents of lutein, β -carotene, β -cryptoxanthin, and all-*trans*-violaxanthin were simultaneously increased when the ethylene treatment was performed under red LED light. In general, consumers eat the pulp of citrus fruits. In the recent years, however, citrus peel becomes more popular in human diet, for example it is added fresh as zest, 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.

ACKNOWLEDGEMENTS

This work was supported by Japanese Society for the Promotion of Science (JSPS; postdoctoral fellowship no. P12395 to Gang Ma), and Grant-in-Aid for Challenging Exploratory Research (25660023).

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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° . B, CCI. C, The photos of the citrus fruit on the sixth day after harvest. H° =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
<i>CitPSY</i>	Sense: CGTTGATGGGCCTAATGCTT Antisense :ACCTGGACTCCCACCTGTCTAA	ACACATAACTCCAACAGC
<i>CitPDS</i>	Sense: TGGCAACCCCCCAGAGA Antisense: CACCCAGTGACTGAATGTGTT	ACTTTGCTTGCCTATTGT
<i>CitZDS</i>	Sense: AAAGGCACTTGTTGATCCTGATG Antisense: ACCAATCAGAGAAGCTTATACTATCCA	CCTTGAAGGACATACGAGAT
<i>CitCRTISO</i>	Sense: AAAGACACACCGGCGGTATC Antisense: CGAGGCATTGGCCCATAG	AGCTCGCGATCAGG
<i>CitLCYb1</i>	Sense: TGGTACCGCTGGGATGGT Antisense: CAATAGGAGCCGCAGCTAAAGT	CACCCTTCAACTGGCT
<i>CitLCYb2</i>	Sense: CCTTGGCTCAACCAGGATGA Antisense: ACCCATTCACACTTTCTGATGA	CAGAGGCAGGCCAC
<i>CitLCYe</i>	Sense: AAGGTGTGTCGAGTCAGGTGTTT Antisense: CCACTGGTAGATTCCGTAATGCT	ATATCTTAGCTCAAAAGTGG
<i>CitHYb</i>	Sense: GCGGCTCACCAGCTTCAC Antisense: CCGAGAAAGAGCCCATATGG	ACTCGGATAAATTCC
<i>CitZEP</i>	Sense: CTAAAGAGCTATGAGAGAGCTAGGAGACT Antisense: CACTGCGGCCGATCTTG	CGAGTGGCTGTTATC
<i>CitVDE</i>	Sense: CAAAGACTTCAATGGGAAGTGGTA Antisense: TGGCAATCAAAAGTATCGAAGGA	TTTCTAGTGGTTTAAATCC

Fig. 1

Fig. 2

Fig. 3

Fig. 4