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Effect of Blue and Red LED Light Irradiation on β-Cryptoxanthin Accumulation in the Flavedo of Citrus Fruits

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Supporting Information

ABSTRACT: β-Cryptoxanthin (β-cry), an antioxidant abundant in citrus fruits, plays an important role in the prevention and treatment of certain diseases, especially cancers. In the present study, to increase the content of β-cry in citrus flavedo, the effects of blue (470 nm) and red (660 nm) light-emitting diode (LED) lights 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 accumulation of β-cry was induced by red light, while it was not affected by blue light. The accumulation of β-cry under red light was attributed to simultaneous increases in the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, and *CitZEP*. The results presented herein might provide new strategies to enhance the commercial and nutritional value of citrus fruits.

KEYWORDS: Blue LED light, citrus, β-cryptoxanthin, flavedo, red LED light

INTRODUCTION

Carotenoids are organic pigments that naturally occur in chromoplasts and chloroplasts of plants and fulfill a variety of critical functions in plants, such as the stabilization of lipid membranes, light harvesting for photosynthesis, and acting as precursors of the plant hormone abscisic acid (ABA) and coloring agents in flowers and fruits.[1−4] Citrus is one of the richest sources of carotenoids in plants. The carotenoid content and composition are critical indexes for the commercial and nutritional value of citrus fruits. Recently, the accumulation of carotenoids in the flavedo and juice sacs of different citrus varieties has been widely investigated.[5−9] Moreover, genes encoding enzymes for the main steps of carotenoid metabolism have been isolated, and their expression has been characterized in citrus fruits (Figure S1 in the Supporting Information). Two isoforms of phytoene synthase (PSY) encoded by different genes, which exhibit differential expression patterns in response to environmental stimuli, have been reported in some plants such as tomato, rice, and maize.[10] However, only one *PSY* cDNA (*CitPSY*) has been isolated from citrus fruits.[11,12] In addition, two cDNA for lycopene β-cyclase (*CitLCYb1* and *CitLCYb2*) have been identified, while for other carotenogenic genes, we have isolated only one cDNA for each gene from citrus fruits.[5,9] During the fruit-ripening process, transcriptional regulation of carotenoid genes appears to be a major mechanism by which the biosynthesis and accumulation of specific carotenoids are regulated. Previously, we found that as fruit maturation progressed, the massive increase in total carotenoid and β,β-xanthophlls in the flavedo and juice sacs was concomitant with the simultaneous induction of gene expression of PSY, phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), lycopene β-cyclase (LCYb), and zeaxanthin expoxidase (ZEP) in Satsuma mandarin and Valencia orange.[5] Carotenoids are not only important to the plants that produce them but also beneficial to human health. Recent studies have identified that the benefits from carotenoids might be due to β-cryptoxanthin (β-cry), a major carotenoid in human blood.[13] Altucci and Gronemeyer reported that β-cry played an important role in the prevention of certain diseases, especially cancers, because of its antioxidative activity.[14] In addition, β-cry served as a retinoic acid receptor (RAR) ligand and exerted beneficial effects on atherogenesis through the activation of RAR.[15] At present, the main dietary source of β-cry is provided from some citrus species, such as Satsuma mandarin and 'Tamami'.[6,16]

Light is an essential environmental factor for plants and influences their growth and development. In higher plants, sensing of light is carried out by various light photoreceptors.[17] Thus, different types of lights might have distinct effects on plant development and the biosynthesis of cell components. Red light is important to the development of the photosynthetic apparatus and may enhance plant growth and development by increasing the net photosynthetic rate.[18,19] Blue light controls the chloroplast development, stomata opening, and shoot growth.[20,21] To date, however, information on the effects of blue and red light on the accumulation carotenoids in citrus is still limited. In the present study, to enhance the content of β-cry in citrus flavedo, the effects of blue (470 nm) and red (660 nm) light-emitting diode (LED) lights on carotenoid accumulation and the expression of genes related to carotenoid biosynthesis were investigated. This study is the first of their kind to investigate effects of the light quality on carotenoid metabolism in citrus fruits and

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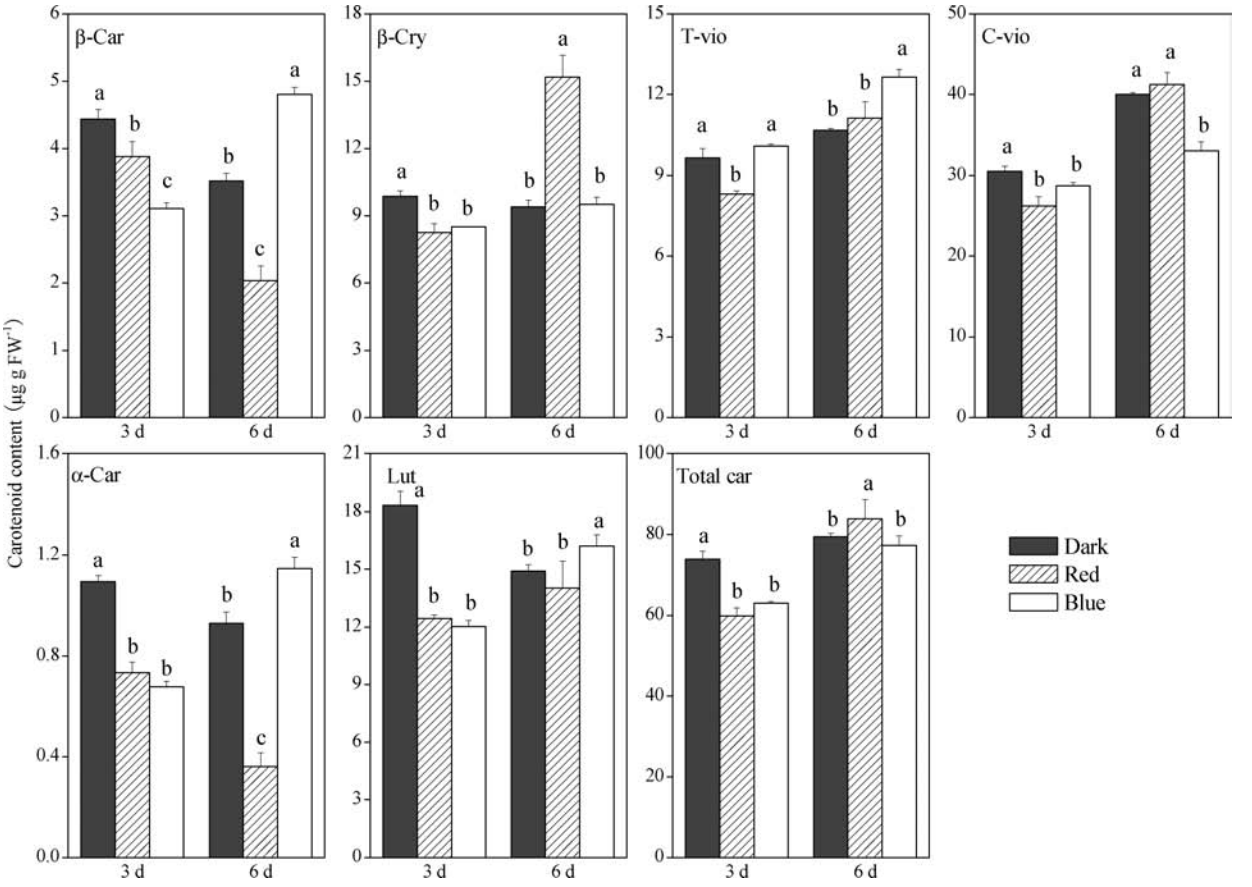

Figure 1. Effect of blue and red LED lights on the carotenoid content in the flavedo of citrus fruits. β-Car, β-carotene. β-Cry, β-cryptoxanthin. T-vio, all-*trans*-violaxanthin. C-vio, 9-*cis*-violaxanthin. α-Car, α-carotene. Lut, lutein. Total car, Total carotenoids. The value for total carotenoids 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.

might provide new strategies to enhance β-cry production in the flavedo of citrus fruits.

MATERIALS AND METHODS

Plant Materials. Fruits of Satsuma mandarin (*Citrus unshiu* Marc.) were harvested in October at the National Institute of Fruit Tree Science, Department of Citrus Research, Okitsu (Shizuoka, Japan). Fruits 45−50 mm in diameter and light green in color were used as materials.

LED Light Irradiation. Fruits were irradiated with blue (470 nm) and red (660 nm) LED lights at an intensity of 50 μmol m^{-2} s^{-1} for 6 days at 20 °C. Fruits 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 use.

Extraction and Determination of Carotenoids. The identification, extraction, and quantification of carotenoid in citrus have been described previously.[5] α-Carotene (α-car), β-carotene (β-car), β-cry, all-*trans*-violaxanthin (T-vio), 9-*cis*-violaxanthin (C-vio), and lutein (Lut) were quantified in the flavedo of Satsuma mandarin during the experimental period. The contents of carotenoids were expressed as μg g^{-1} fresh weight. The carotenoid quantification was performed in three replicates.

Total RNA Extraction and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from the flavedo of Satsuma mandarin fruits according to a previously reported method.[5] 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*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, *CitZEP*, *CitNCED2*, and *CitNCED3* were designed according to Kato et al [7] and Alquézar et al.[9] (Table S1 in the Supporting Information). 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 manufacturer'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 ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems) and normalized with the results of 18S rRNA. Real-time quantitative RT-PCR was performed in three replicates for each sample.

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

RESULTS AND DISCUSSION

Effects of Blue and Red LED Lights on Carotenoid Content and Composition. Citrus fruits were irradiated with blue and red LED lights for 6 days, and changes in the content and composition of carotenoids in the flavedo were examined every 3 days.

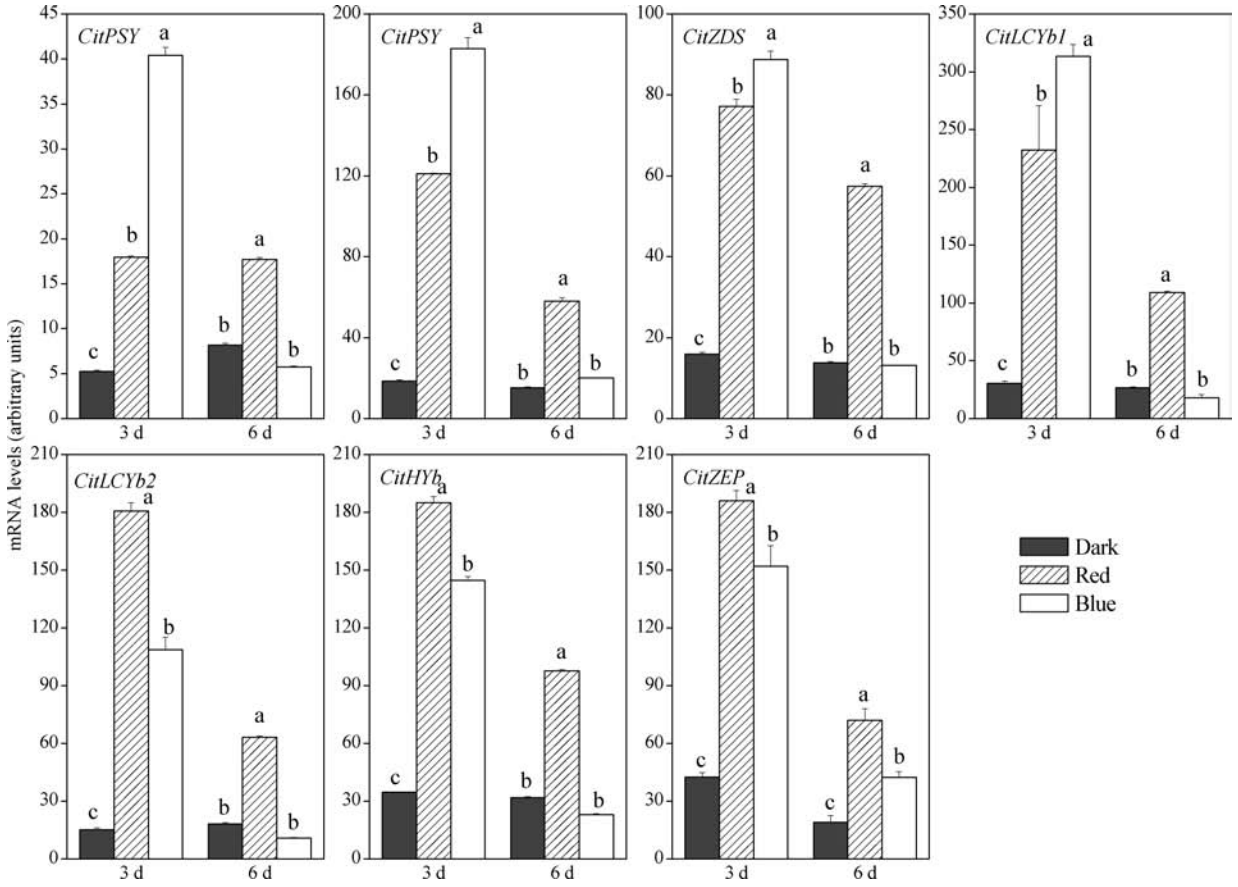

Figure 2. Effect of blue and red LED lights on the expression of carotenoid metabolism-related genes in the flavedo of citrus fruits. The mRNA levels were analyzed by TaqMan real-time quantitative RT-PCR. Real-time RT-PCR amplification of 18S rRNA was used to normalize the expression of the genes under identical conditions. Columns and bars represent the means and SEs ($n = 3$), respectively. Different letters indicate significant differences at the 5% level by Tukey's HSD test.

During the experimental period, the citrus peel gradually turned yellow in the control, as well as the red light and blue light-treated groups. Under red light, the contents of β-car, β-cry, T-vio, C-vio, α-car, and Lut decreased, and the total carotenoid content was lower than the control on the third day (Figure 1). On the sixth day, the contents of β-car and α-car decreased, but the content of β-cry, the predominant carotenoid accumulated in Satsuma mandarin, significantly increased along with an increase in the total carotenoid content. Under blue light, the contents of β-car, β-cry, C-vio, α-car, Lut, and total carotenoids decreased on the third day. On the sixth day, the contents of β-car, T-vio, α-car, and Lut slightly increased, but the contents of β-cry and total carotenoid were kept almost unchanged.

Wu et al. reported that the β-car content was much higher in the red light-treated group than blue light-treated group in leaves and stems of pea seedlings.[19] In tomatoes, the accumulation of lycopene along with an increase in total carotenoid content was also observed in response to red light treatment.[22−24] It has been confirmed that phytochromes mediated red light-induced carotenoid biosynthesis in tomatoes during natural ripening.[22] In the present study, we found that irradiation with red light at an intensity of 50 μmol m^{-2} s^{-1} for 6 days was effective in enhancing the content of β-cry in Satsuma mandarin. LED lights of similar intensity were also applied to increase stem length in chrysanthemum (50 μmol $m^{-2}s^{-1}$), and the growth of grape plantlets (45−55 μmol m^{-2} s^{-1}).[25,26] β-Cry, an antioxidant abundant in citrus fruits, plays an important role in the prevention and treatment of certain cancers.[14] In Japan, to reduce the fruit load and obtain high quality fruits, fruit thinning is performed every year before citrus fruits fully ripen. Although these unripe fruits have little market value, they are useful for the extraction of β-cry, which is rich in the flavedo. The results herein indicated that the application of red light might be an effective method to enhance the utilization of the unripe fruits collected by thinning for the production of β-cry.

Effects of Blue and Red LED Lights on Gene Expression Related to Carotenoid Metabolism. In citrus, transcriptional regulation of carotenoid genes has been reported to be a major mechanism by which the biosynthesis and accumulation of specific carotenoids are regulated during the ripening process.[5,6] To elucidate the mechanism responsible for the high level of β-cry induced by the red light treatment, the expression of genes related to carotenoid metabolism was analyzed. As shown in Figure 2, the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, and *CitZEP*, which make up a set of genes involved in producing β,β-xanthophylls, was simultaneously up-regulated by the red light treatment on the third and sixth days. On the sixth day, the simultaneous increases in the expression of genes involved in β,β-xanthophylls biosynthesis were well consistent with the accumulation of β-cry in the red light treatment. In contrast to red light, blue light had only a temporary effect on the expression of genes related to carotenoid metabolism. In the

blue light treatment, the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, and *CitZEP* was up-regulated on the third day. However, on the sixth day, the expression of these genes was not affected. This temporary up-regulation of carotenogenic gene expression did not cause carotenoids to accumulate after irradiated with blue light for 6 days. In addition, on the third day, although the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, and *CitZEP* was up-regulated by both blue and red light treatments, noticeable increases in the gene expression did not lead to a rapid accumulation of carotenoids. It has been reported that 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in the methylerythritol-4-phosphate (MEP) pathway, which is the upstream pathway for carotenoid biosynthesis, was a rate-limiting enzyme for carotenoid accumulation. In tomato fruits, an increase in the expression of *PSY1* preceded that of *DXS* in the early stages of ripening.[27] In the juice sacs of 'Kiyomi' tangor, noticeable increases in the expression of carotenoid biosynthetic genes occurred before the increase in the expression of *DXS*.[7] In the present study, it is possible that the lower expression level of *DXS* might have limited the accumulation of carotenoids in citrus on the third day in red and blue treatment. Further research into the regulation of the expression of genes in the MEP pathway in response to red and blue light is needed.

In conclusion, carotenoid metabolism was investigated in response to blue and red LED lights in the flavedo of Satsuma mandarin. The results showed that the accumulation of β-cry was induced by red light, while it was not affected by blue light. The accumulation of β-cry under red light was attributed to the simultaneous increases in the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, and *CitZEP*. The results presented herein provide more insights into the regulatory mechanism of carotenoid metabolism in citrus, which might facilitate to enhance the commercial and nutritional value of citrus fruits.

■ ASSOCIATED CONTENT

Supporting Information. Carotenoid biosynthetic pathway in citrus (Figure S1) and primer sequences and TaqMan MGB probes used for the quantitative RT-PCRs of the carotenogenic genes (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ABA, abscisic acid; C-vio, 9-*cis*-violaxanthin; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; LCYb, lycopene β-cyclase; Lut, lutein; MEP, methylerythritol-4-phosphate; PDS, phytoene desaturase; PSY, phytoene synthase; RAR, retinoic acid receptor; T-vio, all-*trans*-violaxanthin; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin expoxidase; α-car, α-carotene; β-car, β-carotene; β-cry, β-cryptoxanthin

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