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Abstract

In the present study, the effects of blue LED light intensity on carotenoid accumulation and expression of genes related to carotenoid biosynthesis were investigated in the juice sacs of Satsuma mandarin (*Citrus unshiu* Marc.) and Valencia orange (*Citrus sinensis* Osbeck) *in vitro*. The results showed that 100 μmol m^{-2} s^{-1} blue LED light (100B) was effective for increasing carotenoid content, especially β-cryptoxanthin, in Satsuma mandarin after cultured *in vitro* for four weeks. In Valencia orange, in contrast, 50 μmol $m^{-2}s^{-1}$ blue LED light (50B) treatment was effective for inducing carotenoid accumulation through increasing the contents of two major carotenoids, all-*trans*-violaxanthin and 9-*cis*-violaxanthin. In addition, gene expression results showed that the simultaneous increases in the expression of genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, and *CitHYb*) involved in producing β,β-xanthophylls were well consistent with the accumulation of β-cryptoxanthin in Satsuma mandarin under 100B, and violaxanthin in Valencia orange under 50B. The results presented herein contribute to further elucidating the regulatory mechanism of carotenoid accumulation by blue LED light.

Keywords: Blue LED light, Carotenoid, Citrus, Juice sacs, Regulatory mechanism

Introduction

Carotenoids are wide-spread organic pigments, and fulfill a variety of critical functions in plants (Cunningham and Ganntt, 1998; Havaux, 1998; Cazzonelli and Pogson, 2010; Nisar et al., 2015). To date, more than 700 carotenoids have been identified in nature of which mainly 6 carotenoids are detected at a high level in human plasma, including β-carotene, α-carotene, lutein, lycopene, β-cryptoxanthin and zeaxanthin (Al-Delaimy et al., 2005). The plasma carotenoid level is associated with less likely risks of cardiovascular diseases, cancers and age related diseases (Khachik et al., 2006; Pouchieu et al., 2014). Recently, some epidemiological studies have shown that the β-cryptoxanthin is one of the most important carotenoids in human plasma; dietary intake of β-cryptoxanthin from citrus fruits can reduce the risks of certain diseases, especially cancers, diabetes and rheumatism because of its antioxidative activity (Cerhan et al., 2003; Yamaguchi et al., 2006; Sugiura et al., 2011; Takayanagi et al., 2011; Yamaguchi 2012; Iskandar et al., 2013). In the past 10 years, to meet the growing demand of consumers for the carotenoid-rich diets, carotenoid biosynthesis in plants has been extensively investigated, and several efforts have been devoted to enhancing carotenoid concentration by genetic modification (Paine et al., 2005; Naqvi et al., 2009; Welsch et al., 2010; Huang et al., 2013).

Citrus fruits, one of the most economically important fruits grown in temperate regions, are a rich and complex source of carotenoids. In citrus, carotenoids are the pigments responsible for the external and internal coloration of fruits, and their contents and compositions are important indexes for the quality of fruits. In the recent years, the accumulation of carotenoid in fruits of various citrus varieties has been studied,

showing that the composition of carotenoid in juice sacs vary greatly among different varieties during the ripening process (Kato et al., 2004; Rodrigo et al., 2004, 2007; Ríos et al., 2010; Ma et al., 2013; Wei et al., 2014). In Satsuma mandarin (*Citrus unshiu* Marc.), β-cryptoxanthin is predominantly accumulated in the juice sacs (Fig. 1). In Valencia orange (*Citrus sinensis* Osbeck), in contrast, the content of β-cryptoxanthin is quite low, and violaxanthin is the major carotenoid in the juice sacs (Fig. 1). It has been proven that the differences in the carotenoid composition between Satsuma mandarin and Valencia orange were attributed to their different gene expression profiles (Kato et al., 2004, 2006). In Satsuma mandarin, the expression levels of the upstream synthesis genes (*CitPSY*, *CitPDS*, *CitZDS*, and *CitLCYb1*) were higher than those in Valencia orange. In contrast, the expression levels of downstream synthesis genes (*CitHYb* and *CitZEP*) were lower in Satsuma mandarin than those in Valencia orange. In addition, β-carotene is converted to zeaxanthin via β-cryptoxanthin by a two-step hydroxylation, which is catalyzed by HYb. In a previous study, we reported that CitHYb catalyzed predominantly the first step conversion by the high substrate specificity of HYb to β-carotene. In Satsuma mandarin, the gene expression of CitHYb increased with a peak in December, which led to the accumulation of β-cryptoxanthin (from 1 μg g^{-1} in August to 15 μg g^{-1} in December) during the ripening periods (Kato et al., 2004).

In citrus fruits, carotenoid metabolism is a complicated process, which is regulated by developmental stages and environmental conditions (Kato et al., 2004; Zhang et al., 2012; Rodrigo et al., 2013). As the growing conditions on trees are not uniform, it is difficult to evaluate the effects of environmental factors on carotenoid accumulation in fruits ripening on tree. In our previous study, a culture system was set up using the juice sacs of Satsuma mandarin and Valencia orange, in which the juice sacs enlarged

gradually with carotenoid accumulation and no callus formed during cultured *in vitro* for eight weeks (Zhang et al., 2012). In this system, blue LED light irradiation was effective for inducing carotenoid accumulation, while red LED light irradiation did not significantly affect the carotenoid content in the juice sacs of Satsuma mandarin and Valencia orange (Zhang et al., 2012). Except for light quality, light intensity was also a key factor regulating carotenoid metabolism in plants (Demmig-Adams, 1992； Bohne and Linden, 2002; Bramley, 2002; Keyhaninejad et al., 2012; Lado et al., 2015). In the present study, to further elucidate the roles of blue light in regulating carotenoid accumulation, the effects of the intensity of blue LED light on carotenoid biosynthesis and expression of the carotenoid biosynthetic genes were investigated in the juice sacs of Satsuma mandarin and Valencia orange *in vitro*. In general, blue light can not penetrate the peel in an intact fruit, thus it is difficult to evaluate the effects of blue light on carotenoid accumulation of the juice sacs using the intact fruit. In this study, we treated the juice sacs directly with different intensities of blue LED light, the results presented herein will contribute to a better understanding of the differential blue-light regulated accumulation of various carotenoids between Satsuma mandarin and Valencia orange.

Materials and methods

Plant Materials

Satsuma mandarin (*Citrus unshiu* Marc.) and Valencia orange (*Citrus sinensis* Osbeck) in the mature green stage were used as materials. The samples were harvested at the National Institute of Fruit Tree Science, Department of Citrus Research, Okitsu

(Shizuoka, Japan).

In vitro culture system and treatments

The fruits were surface-sterilized by a 10-min soak in 70% ethanol, a 30-min soak in 1% (w/v) NaOCl, and rinsed in sterile water. Juice sacs excised from the equatorial region of the fruit were placed on 10 mL of agar medium in culture tubes (22 × 120 mm) and incubated in the dark at 25 °C. The explants were placed with the endocarp side up, so that the juice sacs were not in contact with the Murashige and Skoog (MS) medium supplemented with 10% (w/v) sucrose and 1% (w/v) agar. The pH of the MS medium was adjusted to 5.7 and autoclaved (Zhang et al., 2012). The juice sacs were irradiated with blue (470 nm) LED light at an intensity of 50 μmol m^{-2} s^{-1} (50B) and 100 μmol m^{-2} s^{-1} (100B) for four weeks. During the experimental periods, juice sacs cultured in the dark were used as the control. At each sampling point, 50 juices sacs from 5 fruits were used for each treatment. After each treatment, the juice sacs were immediately frozen in liquid nitrogen, ground into granules and kept at – 80 °C until used.

Extraction and determination of carotenoids

The identification, extraction and quantification of carotenoids in citrus fruits have been described previously (Kato et al., 2004). The contents of β-carotene, β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, α-carotene, and lutein in the juice sacs were examined every two week during the experimental periods. The content of carotenoid was expressed as μg g^{-1} fresh weight. Carotenoid quantification was performed in three replicates. Ratio of β,ε-carotenoids and β,β-carotenoids was calculated as the sum of α-carotene and lutein divided by the sum of β-carotene, β-cryptoxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin.

Total RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from the juice sacs of Satsuma mandarin and Valencia orange 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*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, *CitZEP* and *CitVDE* were designed according to Ma et al. (2013). 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 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 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.

Statistical Analysis

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

Results

Effect of the intensity of blue LED light on carotenoid content and composition

In the present study, the effects of blue LED light intensity on the accumulation of the main carotenoids in the juice sacs were investigated. In Satsuma mandarin, total carotenoid content under 50B or 100B was 2-3 fold that of the control during the experimental periods (Fig. 2A). In the second week, the contents of β-carotene, β-cryptoxanthin, 9-*cis*-violaxanthin, α-carotene, lutein and total carotenoid were lower in the 100B treatment than those in the 50B treatment in Satsuma mandarin. In the fourth week, the contents of β-carotene, β-cryptoxanthin, all-*trans*-violaxanthin, α-carotene, and lutein were significantly increased by the treatment of 100B, and as a result the total carotenoid content was higher in the 100B treatment than that in the 50B treatment. In addition, with the increase in the blue LED light intensity, the ratio of β,ε-carotenoids and β,β-carotenoids increased from 0.17 to 0.28 in Satsuma mandarin in the fourth week (Supplemental Table S1).

In Valencia orange, total carotenoid content increased gradually in the 50B treatment during the experimental periods. With the 100B treatment, the total carotenoid content increased in the first two weeks, and kept constant thereafter. In the second week, the content of β-cryptoxanthin increased, while the contents of β-carotene, 9-*cis*-violaxanthin, α-carotene, lutein and total carotenoid content decreased when the intensity of blue LED light treatment increased from 50B to 100B in Valencia orange. In the fourth week, the contents of β-carotene, β-cryptoxanthin, and lutein were similar between the 50B and 100B treatments. However, the contents of two major carotenoids of Valencia orange, all-*trans*-violaxanthin and 9-*cis*-violaxanthin, were much higher in the 50B treatment than those in the 100B treatment in the fourth week (Fig. 2B). Similar to Satsuma mandarin, the ratio of β,ε-carotenoids and β,β-carotenoids increased (from

0.47 to 0.82) with the increase in the blue LED light intensity in the fourth week (Supplemental Table S1).

Effect of the intensity of blue LED light on gene expression related to carotenoid metabolism

In Satsuma mandarin and Valencia orange, transcriptional regulation of carotenoid biosynthetic genes has been proven to be a major mechanism by which β-cryptoxanthin and violaxanthin is specifically accumulated in the juice sacs during the ripening process (Kato et al., 2004, 2006). As shown in Fig. 3A, the gene expression levels of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, *CitLCYe*, and *CitHYb* were lower in the 100B treatment than those in the 50B treatment in the second week in Satsuma mandarin. In the fourth week, the expression levels of these genes were higher in the 100B treatment than those in the 50B treatment. In Valencia orange, the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, and *CitHYb* was simultaneously down-regulated by 100B treatment compared with the 50B treatment in the second and fourth weeks (Fig. 3B).

Discussion

Blue light is an effective activator of carotenoid accumulation in plants. In the sprouts of Tartary buckwheat (*Fagopyrum tataricum* Gaertn.), the expression levels of genes related to the carotenoid biosynthesis increased under the blue light irradiation from the 2nd day after sowing, and peaked at the 6th day after sowing (Tuan et al., 2013). Johkan et al. (2010) found that carotenoid content in the leaves of lettuce seedlings was increased by blue LED light treatment, and the elevated content of carotenoid in seedlings by blue LED light might have an advantage in light absorption

and contribute to improving photosynthesis of lettuce plants after transplantation. In citrus fruits, we previously reported that blue LED light was effective for inducing carotenoid accumulation in the juice sacs of both Satsuma mandarin and Valencia orange. In the present study, however, the results showed that the effects of blue LED light intensity on carotenoid accumulation in the juice sacs were different between these two citrus varieties. In Satsuma mandarin, the effects of blue light intensity on the carotenoid accumulation were time-related. In the second week, the juice sacs of Satsuma mandarin exhibited a light saturation at 50B, thus the carotenoids contents and expression levels of carotenoid biosynthetic genes were higher at 50B than those at 100B. While in the fourth week, 100B treatment was more effective for inducing carotenoid biosynthesis. In Satsuma mandarin, β-cryptoxanthin is the major carotenoid accumulated in the juice sacs, and exhibits positive effects on human health, such as bone formation and cancers prevention (Cerhan et al., 2003; Yamaguchi et al., 2006; Takayanagi et al., 2011; Yamaguchi, 2012; Iskandar et al., 2013). In this study, the results showed that the expression of genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, and *CitHYb*) involved in producing β,β-xanthophylls was simultaneously up-regulated by 100B in the fourth week, which led to the content of β-cryptoxanthin was significantly increased by the treatment with 100B (7.83 μg g^{-1}) compared with the control (2.98 μg g^{-1}) in the fourth week. In Valencia orange, 50B treatment was effective for inducing carotenoid accumulation during the experimental periods. Under 50B, the gene expression levels of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, and *CitHYb* was increased, which were well consistent with the accumulation of all-*trans*-violaxanthin and 9-*cis*-violaxanthin at 50B in the fourth week. The results presented in this study suggested that the juice sacs of Satsuma mandarin and Valencia orange exhibited

different responses to the blue light intensity, and the different effects of the blue LED light intensity on carotenoid accumulation between Satsuma mandarin and Valencia orange might be attributed to their different carotenoid composition.

The cyclization of lycopene is a key branch point in the carotenoid biosynthetic pathway in citrus fruits. With the transition from the green stage to the orange stage, the pathway shifts from β,ε-carotenoid synthesis to β,β-carotenoid synthesis (Kato et al., 2004; Inoue et al., 2006). In this study, when the blue light intensity increased from 50B to 100B the ratio of β,ε-carotenoids and β,β-carotenoids increased from 0.17 to 0.28 in Satsuma mandarin, and from 0.47 to 0.82 in Valencia orange, respectively. Moreover, gene expression level of *CitLCYe* was higher in the juice sacs at 100B than that at 50B, which was in parallel with the increase in the ratio of β,ε-carotenoids and β,β-carotenoids in the two citrus varieties. These results suggested that 100B treatment may somehow stimulate the shift from β,β-branch to β,ε-branch of the pathway in citrus fruits.

In conclusion, in the present study the effects of blue LED light intensities on carotenoid accumulation were investigated in the juice sacs of Satsuma mandarin and Valencia orange *in vitro*. The results showed that 100B was effective for inducing the carotenoid accumulation in Satsuma mandarin, while 50B was effective in Valencia orange after four weeks cultured *in vitro*. In addition, gene expression results showed that the simultaneous increases in the expression of genes involved in producing β,β-xanthophylls were well consistent with the accumulation of β-cryptoxanthin in Satsuma mandarin under 100B, and violaxanthin in Valencia orange under 50B. The results presented herein contribute to further elucidating the regulatory mechanism of carotenoid accumulation by blue LED light, which will facilitate the application of blue

LED light in horticulture.

Appendix A. Supplementary data

The following are the supplementary data to this article:

Table S1 Effect of blue LED light intensity on the ratio of β, ε-carotenoids and β,β-carotenoids in the fourth week.

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Figure legends

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

Fig. 2. Effect of the intensity of blue LED light on the carotenoid content in the juice sacs of Satsuma mandarin (A) and Valencia orange (B). Total car, Total carotenoid. β-Car, β-carotene. β-Cry, β-cryptoxanthin. T-vio, all-*trans*-violaxanthin. C-vio, 9-*cis*-violaxanthin. α-Car, α-carotene. Lut, lutein. 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. 3. Effect of the intensity of blue LED light on the expression of carotenoid biosynthetic genes in the juice sacs of Satsuma mandarin (A) and Valencia orange (B).. 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.

Fig. 1

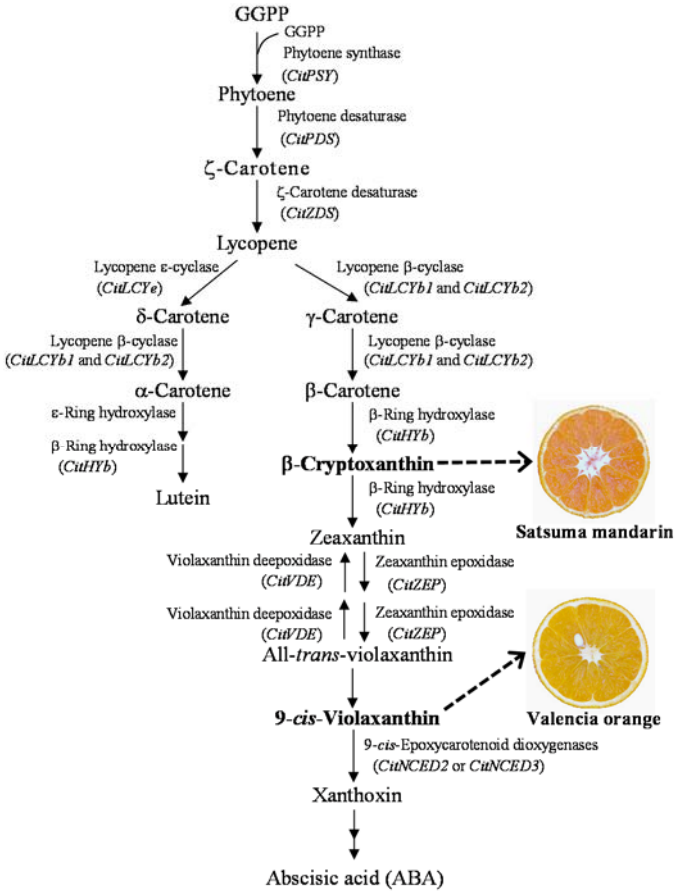
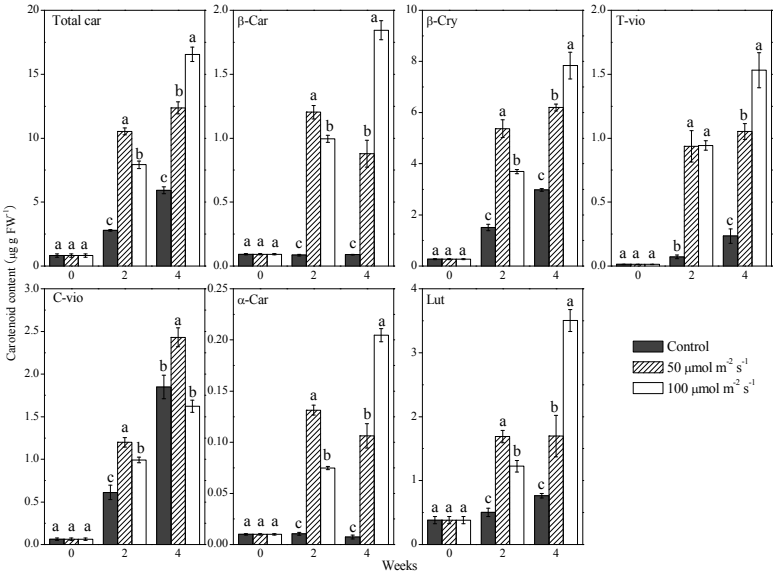

GGPP
GGPP
Phytoene synthase
(CitPSY)
Phytoene
Phytoene desaturase
(CitPDS)
ζ-Carotene
ζ-Carotene desaturase
(CitZDS)
Lycopene
Lycopene ε-cyclase
(CitLCYe)
Lycopene β-cyclase
(CitLCYb1 and CitLCYb2)
δ-Carotene
γ-Carotene
Lycopene β-cyclase
(CitLCYb1 and CitLCYb2)
Lycopene β-cyclase
(CitLCYb1 and CitLCYb2)
α-Carotene
β-Carotene
ε-Ring hydroxylase
β-Ring hydroxylase
(CitHYb)
β-Ring hydroxylase
(CitHYb)
β-Cryptoxanthin
Lutein
β-Ring hydroxylase
(CitHYb)
Satsuma mandarin
Zeaxanthin
Violaxanthin deepoxidase
(CitVDE)
Zeaxanthin epoxidase
(CitZEP)
Violaxanthin deepoxidase
(CitVDE)
Zeaxanthin epoxidase
(CitZEP)
All-trans-violaxanthin
9-cis-Violaxanthin
Valencia orange
9-cis-Epoxycarotenoid dioxygenases
(CitNCED2 or CitNCED3)
Xanthoxin
Abscisic acid (ABA)

Fig. 2

A


Total car
β-Car
β-Cry
T-vio
C-vio
α-Car
Lut
Carotenoid content (μg g FW^{-1})
Weeks
Control
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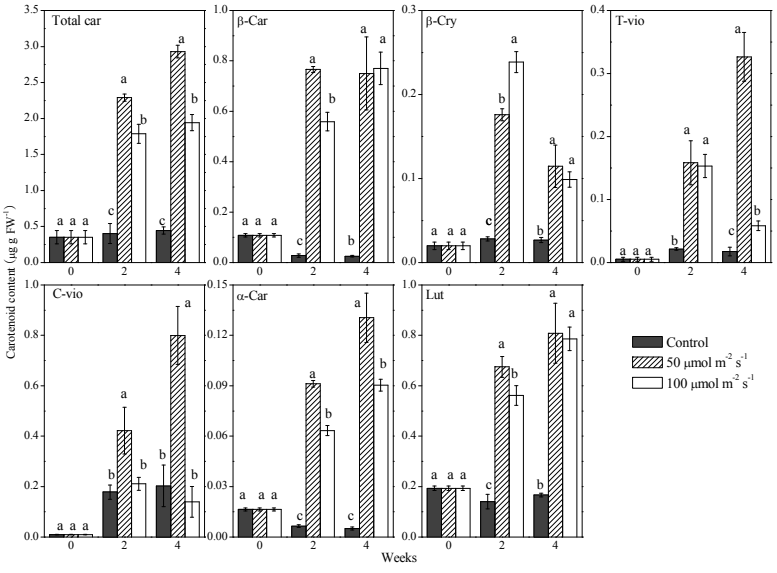
B


Total car
β-Car
β-Cry
T-vio
C-vio
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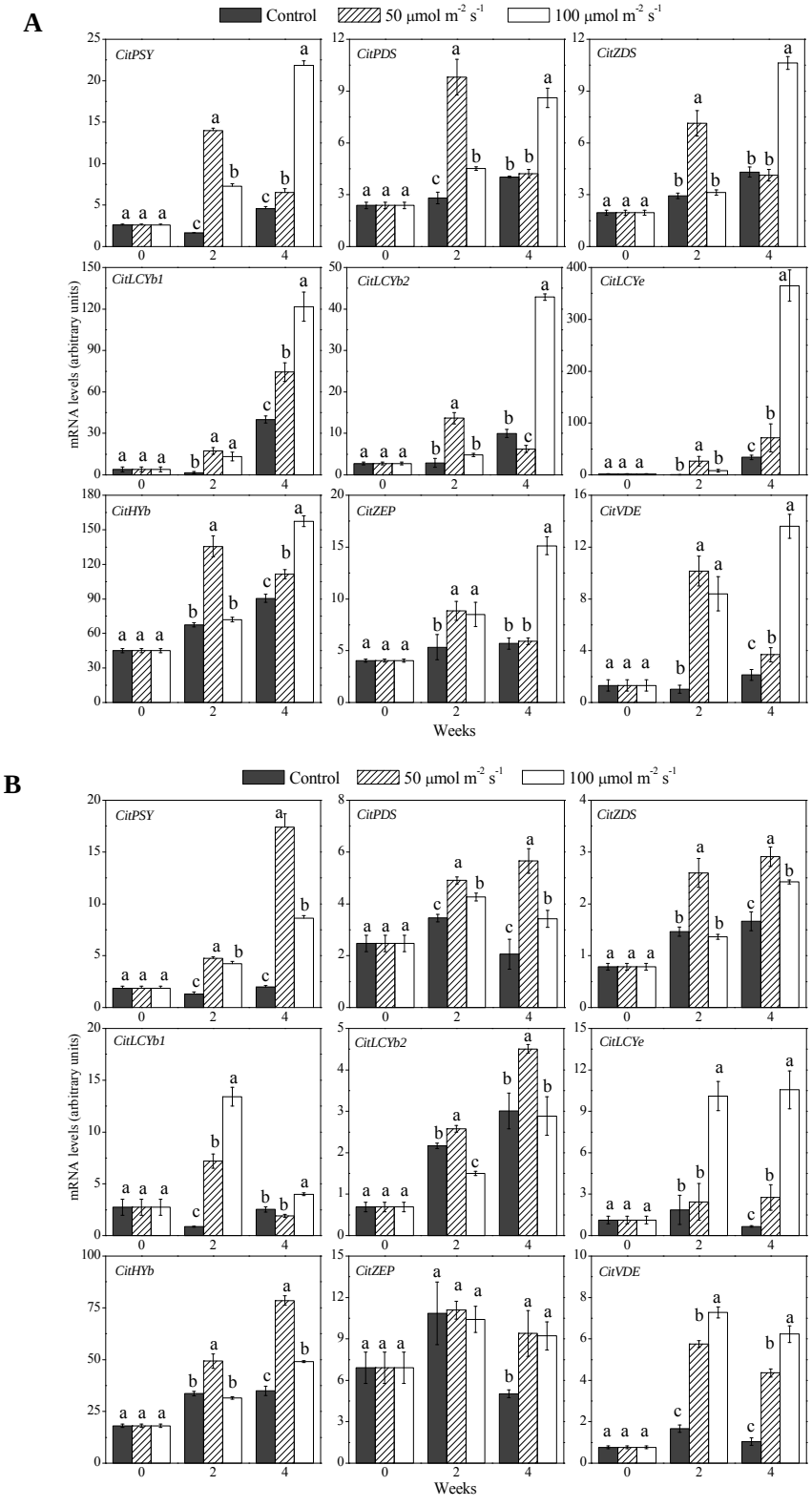
Carotenoid content (μg g FW^{-1})
Weeks
Control
50 μmol m^{-2} s^{-1}
100 μmol m^{-2} s^{-1}

Fig. 3


A
Control
50 μmol m-2 s-1
100 μmol m-2 s-1
CitPSY
CitPDS
CitZDS
CitLCYb1
CitLCYb2
CitLCYe
CitHYb
CitZEP
CitVDE
mRNA levels (arbitrary units)
Weeks
B
Control
50 μmol m-2 s-1
100 μmol m-2 s-1
CitPSY
CitPDS
CitZDS
CitLCYb1
CitLCYb2
CitLCYe
CitHYb
CitZEP
CitVDE
mRNA levels (arbitrary units)
Weeks