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Abstract

In the present study, the effects of red and blue LED lights on the accumulation of ascorbic acid (AsA) were investigated in the juice sacs of three citrus varieties, Satsuma mandarin, Valencia orange, and Lisbon lemon. The results showed that the blue LED light treatment effectively increased the AsA content in the juice sacs of the three citrus varieties, whereas the red LED light treatment did not. By increasing the blue LED light intensity, the juice sacs of the three citrus varieties accumulated more AsA. Moreover, continuous irradiation with blue LED light was more effective than pulsed irradiation for increasing the AsA content in the juice sacs of the three citrus varieties. Gene expression results showed that the modulation of AsA accumulation by blue LED light was highly regulated at the transcription level. The up-regulation of AsA biosynthetic genes (*CitVTC1*, *CitVTC2*, *CitVTC4*, and *CitGLDH*), AsA regeneration genes (*CitMDAR1*, *CitMDAR2*, and *CitDHAR*) and two GSH-producing genes (*CitGR* and *CitchGR*) contributed to these increases in the AsA content in the three citrus varieties.

Key words: AsA, continuous irradiation, pulsed irradiation, regulatory mechanism.

Abbreviations

AsA ascorbic acid

AO ascorbate oxidase

VTC1 GDP-D-mannose pyrophosphorylase (GMP)

GME GDP-D-mannose 3’5-epimerase

VTC2 GDP-L-galactose phosphorylase (GGP)

VTC4 L-galactose-1-phosphate phosphatase (GPP)

GLDH L-galactono-1,4-lactone dehydrogenase

APX ascorbate peroxidase

MDA monodehydroascorbate

MDAR MDA reductase

DHA dehydroascorbate

DHAR DHA reductase

GR glutathione reductase

GSH reduced glutathione

GSSG oxidized glutathione

1. Introduction

Ascorbic acid (AsA), one of the most important antioxidants in plants, protects cells from oxidative stress by interacting enzymatically and non-enzymatically with reactive oxygen species (ROS). AsA is also a key co-factor for various enzymes, and participates in the regulation of photosynthesis, hormone biosynthesis, and senescence in plants [1], [2], [3] and [4]. AsA is as essential to humans as it is to plants. An AsA deficiency can lead to scurvy in humans. Recent studies suggested that AsA effectively reduced the risk of arteriosclerosis, cardiovascular diseases and some cancers [5], [6] and [7]. As there is a mutation in the AsA biosynthetic enzyme L-galactono-1,4-lactone dehydrogenase (GLDH), humans cannot synthesize the AsA in the body and must obtain it through the daily diet. In recent years, to meet health requirements and the growing demand of consumers for highly nutritional foods, AsA metabolism in plants has been extensively investigated, and several efforts have been made to enhance AsA levels in fruits and vegetables [8], [9], [10], [11], [12] and [13].

To date several AsA biosynthetic pathways have been characterized, and of these, L-galactose pathway was identified as the major route for AsA biosynthesis in higher plants [14], [15], [16] and [17]. In the L-galactose pathway, AsA is synthesized from D-mannose-1-phosphate by a series of enzymes, including GDP-D-mannose pyrophosphorylase (GMP, VTC1), GDP-D-mannose 3’5-epimerase (GME), GDP-L-galactose phosphorylase (GGP, VTC2), L-galactose-1-phosphate phosphatase (GPP, VTC4), and GLDH (Fig. 1). As shown in Fig. 1, AsA can be further oxidized by ascorbate peroxidase (APX) and ascorbate oxidase (AO), and regenerated by NADPH-dependent MDA reductase (MDAR) and dehydroascorbate reductase (DHAR) via the ascorbate–glutathione cycle [3], [4] and [18]. In broccoli and tomato, it has been found that the enzymes in the ascorbate-glutathione cycle exist as isoezymes distributed in different cellular organelles such as chloroplasts, plastids, mitochondria, and peroxisomes [11] and [19].

Light is one of the most essential environmental factors for plant growth and development. Red and blue lights are reported to have the greatest impacts on plant growth and development because they are the major energy sources for photosynthesis, and regulate many responses in higher plants [20], [21], [22] and [23]. A previous study demonstrated that blue LED light was more effective for inducing carotenoid accumulation in the juice sacs of citrus fruits than red LED light. PSY, a rate-limiting enzyme for carotenoid biosynthesis, was up-regulated by blue LED light, but was not significantly affected by red LED light [24]. In plants, the AsA metabolic pathway interacts with photosynthetic and respiratory electron transport chains, and the accumulation of AsA is known to be influenced by the quantity and quality of light [25], [26] and [27]. Mastropasqua et al. [28] reported that the AsA content in oat leaf segments significantly decreased in the darkness. This decrease in AsA content could be fully recovered after oat leaf segments had been irradiated with blue light for one day, but not with red light. In broccoli, red LED light effectively suppressed reductions in AsA after harvest, whereas blue LED light had no effect on the metabolism of AsA [12].

Citrus is one of the most economically important fruit grown in temperate regions. Large quantities of citrus fruits are consumed in the world each year, and it is increasingly evident that citrus fruits not only taste good, but are also a good resource of AsA. Even though AsA metabolism has been well characterized in several plants, including the tomato [16], apple [29], kiwifruit [30], and broccoli [18], information on the metabolism of AsA in the citrus fruits during the ripening process and its regulation is still limited [31] and [32]. In the present study, to enhance AsA levels in juice sacs, the regulatory mechanism of AsA metabolism in response to red and blue LED lights were examined in Satsuma mandarin, Valencia orange and Lisbon lemon *in vitro*. This study is the first to investigate the effects of light quality on the metabolism of AsA in citrus fruits.

2. Materials and methods

2.1 Plant Materials

Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*C. sinensis* Osbeck), and Lisbon lemon (*C. limon* Burm.f.) were harvested from the NARO Institute of Fruit Tree Science, Department of Citrus Research, Okitsu (Shizuoka, Japan). Fruit samples were collected periodically between August and January for Satsuma mandarin and between August and February for Valencia orange and Lisbon lemon. The juice sacs were separated from sampled fruit, immediately frozen in liquid nitrogen, ground into granules and kept at -80 ˚C until used.

2.2 Extraction and assays of AsA

The AsA content was assayed by HPLC in accordance with the published methods [12] and [33]. Each frozen sample (1 g) was homogenized using 5 mL of 2% metaphosphoric acid. After centrifuged at 14,000 ×g for 20 min, the supernatant was collected and then filtered through Miracloth (Calbiochem, La Jolla, CA, USA) and a 0.2-μm cellulose acetate filter (Advantec, Tokyo, Japan), respectively. A 20-μL aliquot was analyzed by HPLC with a J'sphere ODS-M80 column (YMC, Kyoto, Japan) and a LC-10AD pump (Shimadzu, Kyoto, Japan). The column was eluted with 80% acetonitrile: 0.04% phosphoric acid at a flow rate of 1.0 mL min^{-1} at 20 °C. AsA was monitored at 245 nm using an SPD-10A spectrophotometric detector (Shimadzu).The content of AsA was calculated as μmol g^{-1} fresh weight, and AsA quantification was performed in three replicates.

2.3 *In vitro* culture system and treatments

The fruit collected in October and cultured *in vitro*. The culture process was according to the method described by Zhang et al. [33]. The whole fruits were soaked in 70% ethanol for 10 min, and then in 1% (w/v) NaOCl for 30 min. After the fruits were washed with sterile water, juice sacs were excised from fruit, and incubated with the

endocarp side up on 10 ml of agar medium in culture tubes (22 × 120 mm) at 25 °C. Murashige and Skoog (MS) supplemented with 10% (w/v) sucrose and 1% (w/v) agar was used as medium. The pH of the MS medium was adjusted to 5.7 and autoclaved [24]. Experimental 1: juice sacs were irradiated with blue (470 nm) and red (660 nm) LED lights at an intensity of 50 μmol $m^{-2}s^{-1}$ for four weeks. Experimental 2: juice sacs were irradiated with blue LED light at an intensity of 50 μmol $m^{-2}s^{-1}$ and 100 μmol $m^{-2}s^{-1}$ for four weeks. Experimental 3: juice sacs were treated with continuous and pulsed blue LED light irradiation at an intensity of 100 μmol $m^{-2}s^{-1}$ for four weeks. A period of 400 μs and a duty of 50% were used for pulsed blue LED light. 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.

2.4 Isolation and sequence analysis of genes related to AsA metabolism

Total RNA was extracted in accordance with the method described by Kato et al. [34]. Two μg of total RNA was used to synthesize the first-strand cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster city, CA, USA).

The cDNA fragments of genes related to AsA metabolism (*CitVTC1*, *CitVTC2*, *CitVTC4*, *CitGLDH*, *CitAPX1*, *CitAPX2*, *CitAPX3*, *CitchAPX*, *CitAO*, *CitMDAR1*, *CitMDAR2*, *CitDHAR*, *CitGR*, and *CitchGR*) were amplified by PCR using a cDNA template and a set of primers was shown in Table 1. The amplified cDNAs were confirmed by sequencing.

2.5 Real-time quantitative RT-PCR

TaqMan MGB probes and sets of primers for *CitVTC1*, *CitVTC2*, *CitVTC4*, *CitGLDH*, *CitAPX1*, *CitAPX2*, *CitAPX3*, *CitchAPX*, *CitAO*, *CitMDAR1*, *CitMDAR2*, *CitDHAR*, *CitGR*, and *CitchGR* were designed on the basis of the common sequences of each gene in citrus using Primer Express software (Applied Biosystems) (Table 2). Gene

expression was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The conditions were according to the methods published before [12] and [24]. 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. Gene expression levels normalized with the values of 18S ribosomal RNA were analyzed with StepOnePlus™ Real-Time PCR System Software (Applied Biosystems). For each sample, real-time quantitative RT-PCR was performed in three replicates.

2.6 Statistical Analysis

All values are shown as the mean ± SE for three replicates. Data were analyzed. Tukey's HSD test (at $P<0.05$) and the Student's *t*-test (at $P<0.05$ and $P<0.01$) were used to compare the treatment means.

3. Results

3.1 Accumulation of AsA during fruit ripening in three citrus varieties

The AsA content was maintained in the juice sacs of Satsuma mandarin throughout the ripening process (Fig. 2). However, it decreased significantly in Valencia orange and Lisbon lemon from August and then increased gradually from October (Fig. 2). Among the three citrus varieties, the AsA content was the lowest in Satsuma mandarin and the highest in Lisbon lemon.

3.2 Expression of genes related to AsA metabolism during fruit ripening in three citrus varieties

In the present study, the cDNAs of AsA biosynthetic genes (*CitVTC1*, *CitVTC2*, *CitVTC4*, and *CitGLDH*), AsA oxidation genes (*CitAPX1*, *CitAPX2*, *CitAPX3*, *CitchAPX*, and *CitAO*), AsA regeneration genes (*CitMDAR1*, *CitMDAR2*, and

CitDHAR), and glutathione reductase genes (*CitGR* and *CitchGR*) were isolated from the Satsuma mandarin, Valencia orange, and Lisbon lemon, and changes in the expression of these genes during the ripening process were analyzed in the three citrus varieties (Fig. 3).

In Satsuma mandarin, the gene expression of *CitVTC1*, *CitVTC2*, *CitVTC4* and *CitGLDH* increased gradually and reached a maximum in October and December. In Valencia orange, the gene expression of *CitVTC1*, *CitVTC2*, and *CitVTC4* decreased rapidly from August to a low level, and subsequently increased gradually during the ripening process. The gene expression of *CitGLDH* increased with a peak in December in Valencia orange. In Lisbon lemon, the gene expression of *CitVTC1* and *CitGLDH* increased gradually during the ripening process. The gene expression of *CitVTC2* and *CitVTC4* in Lisbon lemon increased with a peak in December and October, respectively. The gene expression levels of *CitVTC2* and *CitVTC4* in Satsuma mandarin were lower than those in Valencia orange and Lisbon lemon. In Lisbon lemon, the gene expression levels of *CitVTC2* and *CitGLDH* were higher than those in Satsuma mandarin and Valencia orange during the ripening process.

The gene expression of *CitAPX1*, *CitAPX2*, *CitAPX3*, and *CitchAPX* increased rapidly with a peak during the ripening process in Satsuma mandarin and Valencia orange. In Lisbon lemon, the gene expression of *CitAPX2* and *CitchAPX* increased gradually during the ripening process. The gene expression of *CitAPX1* and *CitAPX3* increased with a small peak during the ripening process in Lisbon lemon. The gene expression of *CitAO* decreased to a markedly low level during the ripening process in the three citrus varieties. In Satsuma mandarin, the gene expression levels of *CitAPX1*, *CitAPX2*, *CitAPX3*, and *CitchAPX* were higher than those in the other two citrus verities.

In Satsuma mandarin and Valencia orange, the gene expression of *CitGR* and *CitchGR* increased with a peak in October and December, respectively. In Lisbon lemon, the gene expression level of *CitGR* and *CitchGR* increased gradually during the ripening

process. The gene expression of *CitMDAR1*, *CitMDAR2*, and *CitDHAR* increased with a peak in September during the ripening process in Satsuma mandarin. In Valencia orange, the gene expression of *CitMDAR1*, *CitMDAR2*, and *CitDHAR* decreased slightly in September, subsequently increased, and reached a maximum in December. In Lisbon lemon, the gene expression of *CitMDAR1*, *CitMDAR2*, and *CitDHAR* increased with a peak in December. In the later stage (from October), the expression levels of *CitMDAR1* and *CitDHAR* were the highest in Lisbon lemon, but the lowest in Satsuma mandarin from October.

3.3 Effects of red and blue LED lights on AsA accumulation in three citrus varieties *in vitro*

As the environmental conditions on trees are not uniform and hard to control, it is difficult to evaluate the effects of light quality on AsA metabolism in the citrus fruits ripening on trees. In a previous study, we successfully established a culture system of citrus juice sacs *in vitro*, in which the juice sacs enlarged gradually without the formation of a callus (24). Using this system, the effects of red and blue LED lights on AsA accumulation in juice sacs were investigated in the present study (Supplemental Fig. S1). As shown in Fig. 4, the red LED light treatment did not affect the accumulation of AsA in the juice sacs of Satsuma mandarin and Lisbon lemon after two- and four- week cultures *in vitro*. In Valencia orange, the red LED light treatment increased the AsA content in the second week, but not in the fourth week. In contrast to red LED light, the blue LED light treatment significantly increased the AsA content in the juice sacs of the three varieties after two- and four- week cultures *in vitro* (Fig. 3).

3.4 Effects of the intensity of blue LED light on AsA accumulation in three citrus varieties *in vitro*

As shown in Fig. 5, AsA accumulation was affected by the intensity of blue LED light in the juice sacs of the three citrus varieties *in vitro*. In Satsuma mandarin and

Lisbon lemon, juice sacs accumulated more AsA with an increase in blue LED light intensity in the second and fourth weeks. In Valencia orange, no significant difference in the AsA content was observed between the 50 μmol $m^{-2}s^{-1}$ and 100 μmol $m^{-2}s^{-1}$ blue LED light treatments in the second week. However, 100 μmol $m^{-2}s^{-1}$ blue LED light treatment induced higher AsA content in the juice sacs of Valencia orange in the fourth week (Fig. 4).

3.5 Effects of continuous and pulsed blue LED light irradiation on AsA accumulation in three citrus varieties *in vitro*

In the present study, the effects of two operational modes of blue LED light (continuous and pulsed) on AsA accumulation were investigated in the juice sacs of the three citrus varieties (Fig. 6). In Satsuma mandarin and Valencia orange, no significant difference in AsA content was detected between continuous and pulsed irradiation of blue LED light in the second week. In the fourth week, the AsA content was higher with the continuous irradiation treatment than that with the pulsed irradiation treatment in Satsuma mandarin and Valencia orange. In Lisbon lemon, the continuous irradiation treatment induced a higher AsA content in the second week, while the pulsed irradiation treatment induced a higher AsA content in the fourth week.

3.6 Effects of blue LED light on the expression of genes related to AsA metabolism

Changes in the expression of AsA metabolic genes in response to continuous irradiation with blue LED light at an intensity of 100 μmol $m^{-2}s^{-1}$ were analyzed in the three citrus varieties. As shown in Fig. 7, the gene expression of *CitVTC1*, *CitVTC2*, *CitVTC4*, and *CitGLDH* was simultaneously up-regulated in the juice sacs of Satsuma mandarin, Valencia orange and Lisbon lemon by the blue LED light treatment in the second and fourth weeks. In Satsuma mandarin, the expression of three cytosolic APX genes (*CitAPX1*, *CitAPX2*, and *CitAPX3*) and one chloroplastic APX gene (*CitchAPX*) was up-regulated by the blue LED light treatment in the second and fourth weeks. In

Valencia orange, the blue LED light treatment down-regulated the gene expression of *CitAPX1*, while it up-regulated the gene expression of *CitAPX2* in the second week. The gene expression of *CitAPX3*, and *CitchAPX* was up-regulated by blue LED light in Valencia orange after two- and four- week cultures *in vitro*. In Lisbon lemon, the gene expression of *CitAPX1* and *CitAPX3* under blue LED light was down-regulated in the second week, while it was up-regulated in the fourth week. The gene expression of *CitAPX2* and *CitchAPX* was up-regulated by blue LED light in Lisbon lemon in the second and fourth weeks. The gene expression of *CitAO* was up-regulated in Satsuma mandarin and Lisbon lemon by the blue LED light treatment in the second and fourth weeks. In Valencia orange, the gene expression of *CitAO* was significantly up-regulated in the second week, while it was slightly down-regulated in the fourth week by the blue LED light treatment. In Satsuma mandarin and Valencia orange, the gene expression of *CitMDAR1* and *CitMDAR2* was up-regulated by the blue LED light in the second and fourth weeks. In Lisbon lemon, the gene expression of *CitMDAR1* and *CitMDAR2* under the blue LED light was down-regulated in the second week, while it was up-regulated in the fourth week. The gene expression of *CitDHAR* was simultaneously up-regulated by the blue LED light treatment in the three citrus varieties in the second and fourth weeks. The expression of cytosolic GR gene (*CitGR*) and chloroplastic GR gene (*CitchGR)* was up-regulated in Satsuma mandarin and Valencia orange by the blue LED light treatment in the second and fourth weeks. In Lisbon lemon, the gene expression of *CitGR* and *CitchGR* was not significantly affected in the second week, while it was up-regulated in the fourth week by the blue LED light treatment.

4. Discussion

4.1 AsA metabolism in three citrus varieties

In fruits, the metabolism of AsA is influenced by geographical origins and growing conditions; therefore, it varies greatly among different species during the ripening

process. Previous studies reported that the AsA content during fruits development and ripening increased in grape berries and guava, decreased in mango, and was maintained in melon [35], [36] and [37]. In citrus, AsA accumulation pattern during ripening process differed among three varieties. In Satsuma mandarin, the AsA content was maintained and markedly lower than those of the other two varieties during the ripening process (Fig. 2). Among the three citrus varieties, the AsA content was the highest in Lisbon lemon during the ripening process (Fig. 2). The three citrus varieties, Satsuma mandarin, Valencia orange, and Lisbon lemon, were grown in the same area under identical growing conditions, and the different patterns of AsA accumulation were observed. Therefore, these three citrus varieties are good materials for investigating the regulation mechanisms of AsA accumulation in citrus fruits.

In the recent years, the expression of genes related to AsA metabolism was extensively studied at the transcription level [16], [30], [38], [39] and [40]. In citrus fruits, *VTC1* (*GMP*), *VTC2* (*GGP*), *VTC4* (*GPP*), and *GLDH* in the L-galactose pathway were identified as the key genes responsible for the regulation of AsA contents in the pulp of citrus fruits during the ripening process [31] and [32]. In addition to its biosynthesis, the ascorbate–glutathione cycle also plays important roles in regulating the accumulation of AsA in the pulp of citrus fruits. Alós et al. [32] reported that increases in the gene expression of *MDAR* contributed to enhancing the turnover of AsA in the pulp of oranges and mandarins during the ripening process. In the present study, changes in the expression of AsA metabolic genes were analyzed in three citrus varieties during the ripening process in order to elucidate why AsA contents differed among these varieties (Fig. 3). The results suggested that the expressional patterns of the AsA metabolic genes varied greatly among the three citrus varieties during the ripening process. In Satsuma mandarin, changes in the expression of AsA biosynthetic genes (*CitVTC1*, *CitVTC2*, and *CitVTC4*), AsA regeneration genes (*CitMDAR1*, *CitMDAR2*, and *CitDHAR*), and AsA oxidation genes (*CitAPX1*, *CitAPX2*, and *CitAPX3*) were similar; simultaneous increases and peaks in the early stage (from August to October)

followed by decreases in the later stage (from October). The similar expression patterns of AsA biosynthetic genes, AsA regeneration genes, and AsA oxidation genes contributed to maintaining the AsA content at a constant level during the ripening process in Satsuma mandarin. In Valencia orange, the gene expression of *CitVTC1*, *CitVTC2*, *CitVTC4*, *CitMDAR1*, *CitMDAR2*, and *CitDHAR* decreased rapidly to a low level from August, and then gradually increased from October. Changes in the expression of AsA biosynthetic genes and regeneration genes occurred concomitantly with the accumulation of AsA during the ripening process in Valencia orange. In Lisbon lemon, the gene expression of *CitAPX1*, and *CitAPX3* increased rapidly from August, which may have led to a decrease in the content of AsA in the early stage. The rapid up-regulation of the gene expression of *CitVTC1*, *CitVTC2*, *CitMDAR1*, and *CitDHAR* from October led to an increase in the AsA content in the later stage in Lisbon lemon. Thus, transcription profiles in biosynthesis, oxidization and recycle processes of AsA were different among three varieties and the transcriptional balances in these processes would cause different accumulation patterns during the ripening process.

4.2 Effects of light quality on AsA metabolism in juice sacs *in vitro*

In higher plants, sensing of blue and red lights is carried out by different light photoreceptors, and blue and red lights produce different morphogenetic and photosynthetic responses in plants [41] and [42]. In tomato, illumination with blue LED light effectively increased fruits yield and led to quality improvements as well as enhanced disease resistance, while illumination with red LED light induced the accumulation of lycopene along with an increase in total carotenoid content [22], [43], [44] and [45]. Light quality is also important for AsA biosynthesis and catabolism in plants [27] and [28]. In broccoli, reductions in AsA after harvest were suppressed by red LED light, but not by blue LED light [12]. In contrast to broccoli, blue LED light was more effective than red LED light for enhancing the AsA content in the juice sacs of citrus fruits (Fig. 4), and AsA content increased in proportion to blue LED light

intensity (Fig. 5). This response to blue and red LED light was similar to carotenoid accumulation [42]. These results suggested that the regulation of AsA metabolism in response to blue and red LED lights was plant species-dependent.

Pulsed light is a nonthermal emerging technology, and exhibits high penetration, emission capacity, and peak power distribution due to the several times amplification of energy during its production. In recent years, pulsed light has been widely used for the rapid inactivation of microorganisms on food surfaces [46] and [47]. However, research on the effects of pulsed light on fruit quality is still limited. Charles et al. [48] reported that a pulsed light treatment enhanced the carotenoid content, but not the AsA content in freshly cut mangoes. The results of the present study showed that the pulsed blue LED light treatment led to higher AsA contents in the juice sacs of the three citrus varieties than the control (Fig. 5). Although continuous irradiation of blue LED light is superior to pulsed light for increasing AsA content, the pulsed blue LED light has practical effect on it and would be applied as an alternative operation.

4.3 Effects of blue LED light on the expression of genes related to AsA metabolism

Recent studies have suggested that VTC2 is a key enzyme controlling AsA biosynthesis, and also that the light-induced accumulation of AsA was mainly mediated by an increase of *VTC2* expression [49] and [50]. In the present study, the expression of AsA metabolic genes was investigated in the juice sacs of Satsuma mandarin, Valencia orange, and Lisbon lemon under continuous irradiation of 100 μmol $m^{-2}s^{-1}$ blue LED light. The results showed that the gene expression of *CitVTC1*, *CitVTC2*, *CitVTC4*, and *CitGLDH* was up-regulated simultaneously in the juice sacs of the three citrus varieties by the blue LED light treatment (Fig. 7). These increases in the expression of AsA biosynthetic genes were consistent with the higher AsA content with the blue LED light treatment in the juice sacs of the three citrus varieties.

In plants, APX is the key protective enzyme of the antioxidant system. The expression of APX genes is activated by environmental stimuli, such as drought,

high-intensity light, high temperature, and salt stress. Previous studies reported that the gene expression of *APX2* was rapidly induced under photo-oxidative stress [51] and [52]. The results of the present study showed that the gene expression of *CitAPX2* and *CitchAPX* was simultaneously up-regulated in the juice sacs of the three citrus varieties by the blue LED light treatment (Fig. 7). The increase observed in the expression of *APX* contributed to protecting the juice sacs against oxidative stress under blue LED light. These results are consistent with previous findings in the broccoli treated with modified white light [12]. In addition, the down-regulation of *CitAPX1* by blue LED light in Valencia orange and Lisbon lemon contributed to increasing the AsA content in these two citrus varieties.

MDAR and DHAR are the enzymes responsible for AsA regeneration in plants. AsA levels can be elevated in plants by increasing the gene expression of MDAR and DHAR [8] and [53]. In broccoli, the up-regulation of *BO-MDAR1*, *BO-MDAR2*, and *BO-DHAR* contributed to maintaining a high AsA content after harvest with an electrostatic atomization treatment [11]. In the present study, the results showed that the gene expression of *CitMDAR1* and *CitMDAR2* was up-regulated by blue LED light in Satsuma mandarin and Valencia orange in the second and fourth weeks. In Lisbon lemon, the gene expression of *CitMDAR1* and *CitMDAR2* was up-regulated by blue LED light in the fourth week. The simultaneous up-regulation of the gene expression of *CitDHAR* was observed in the three citrus varieties treated with blue LED light in the second and fourth weeks. These results suggested that the increases in the expression of AsA regeneration genes enhanced AsA contents in the juice sacs of the three citrus varieties treated by blue LED light.

As shown in Fig. 1, AsA is regenerated from DHA by DHAR using GSH as an electron donor. GSSG is then converted back to the reduced form GSH by NADPH-dependent GR [18]. Two genes that encode GR have been identified in higher plants; one is predicted to encode a cytosolic GR, while the other is predicted to encode a chloroplastic GR. Previous studies reported GR activities in the cytosol and

chloroplast differed [54] and [55]. In the present study, the gene expression of *CitGR* and *CitchGR* was up-regulated in Satsuma mandarin and Valencia orange by blue LED light treatment in the second and fourth weeks. In Lisbon lemon, the gene expression of *CitGR* and *CitchGR* was up-regulated in the fourth week by blue LED light. GR provided the GSH supply for DHAR to regenerate AsA; thus, the up-regulation of *GR* expression also contributed to increases in the AsA content with the blue LED light treatment in the three citrus varieties.

5. Conclusion

In the present study, the effects of red and blue LED lights on AsA accumulation were investigated in the juice sacs of three citrus varieties, Satsuma mandarin, Valencia orange, and Lisbon lemon. The results showed that red LED light did not affect AsA metabolism in the three citrus varieties. In contrast, continuous irradiation with blue LED light at an intensity of 100 μmol $m^{-2}s^{-1}$ effectively enhanced the content of AsA in the three citrus varieties. Additionally, the modulation of AsA accumulation by blue LED light was highly regulated at the transcription level. The up-regulation of AsA biosynthetic genes (*CitVTC1*, *CitVTC2*, *CitVTC4*, and *CitGLDH*), AsA regeneration genes (*CitMDAR1*, *CitMDAR2*, and *CitDHAR*), and two GSH producing genes (*CitGR* and *CitchGR*) led to an increase in the AsA content in the three citrus varieties. As the juice sacs are the major parts of the pulp, the results obtained herein will provide a theoretical basis for the application of blue LED light to enhance AsA contents in citrus fruits.

Reference

[1] M.W. Davey, M. Van Montagu, D. Inzed, M. Sanmartin, A. Kanellis, N. Smirnoff, I.J.J. Benzie, J.J. Strain, D. Favell, J. Fletcher, Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of procession, J. Sci. Food Agric.

80 (2000) 825-860.

[2] N. Smirnoff, P.L. Conklin, F.A. Loewus, Biosynthesis of ascorbic acid in plants: a renaissance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 437-467.

[3] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant sci. 7 (2002) 405-410.

[4] T. Mori, H. Terai, N. Yamauchi, Y. Suzuki, Effects of postharvest ethanol vapor treatment on the ascorbate-glutathione cycle in broccoli florets, Postharvest Biol. Technol. 52 (2009) 134-136.

[5] A.T. Diplock, J.L. Charleux, G. Crozier-Willi, F.J. Kok, C. Rice-Evans, M. Roberfroid, W. Stahl, J. Viña-Ribes, Functional food science and defense against reactive oxidative species, Br. J. Nutr. 80 (1998) 77-112.

[6] J. Du, J.J. Cullen, G.R. Buettner, Ascorbic acid: chemistry, biology and the treatment of cancer, Biochim. Biophys. Acta 1826 (2012) 443-457.

[7] K.B. Felipe, J. Benites, C. Glorieux, B. Sid, M. Valenzuela, M.R. Kviecinski, R.C. Pedrosa, J.A. Valderrama, P. Levêque, B. Gallez, J. Verrax, P. Buc Calderon, Antiproliferative effects of phenylaminonaphthoquinones are increased by ascorbate and associated with the appearance of a senescent phenotype in human bladder cancer cells, Biochem. Biophys. Res. Commun. 433 (2013) 573-578.

[8] Z. Chen, T.E. Youong, J. Ling, D.R. Gallie, Increasing vitamin C content of plants through enhanced ascorbate recycling, Proc. Natl. Acad. Sci. USA 100 (2003) 3525-3530.

[9] R.D. Hancock, R. Viola, Improving the nutritional value of crops through enhancement of L-ascorbic acid (vitamin C) content: rationale and biotechnological opportunities, J. Agric. Food Chem. 53 (2005) 5248-5257.

[10] G. Ma, L.C. Zhang, M. Kato, K. Yamawaki, T. Asai, F. Nishikawa, Y. Ikoma, H. Matsumoto, Effect of 1-methylcyclopropene on the expression of genes for ascorbate metabolism in postharvest broccoli, Postharvest Biol. Technol. 58 (2010) 121-128.

[11] G. Ma, L.C. Zhang, M. Kato, K. Yamawaki, T. Asai, F. Nishikawa, Y. Ikoma, H. Matsumoto, T. Yamauchi, T. Kamisako, Effect of electrostatic atomization on ascorbate metabolism in postharvest broccoli, Postharvest Biol. Technol. 74 (2012) 19-25.

[12] G. Ma, L.C. Zhang, K. Yamawaki, T. Asai, F. Nishikawa, S. Maezawa, H. Sato, N. Kanemitsu, M. Kato, Effect of red and blue LED light irradiation on ascorbate content and expression of genes related to ascorbate metabolism in postharvest broccoli, Postharvest Biol. Technol. 94 (2014) 97-104.

[13] D.R. Gallie, L-ascorbic Acid: a multifunctional molecule supporting plant growth and development. Scientifica (Cairo) 2013 (2013) 795964.

[14] G.L. Wheeler, M.A. Jones, N. Smirnoff, The biosynthetic pathway of vitamin C in higher plants, Nature 393 (1998) 365-369.

[15] C.L. Linster, S.G. Clarke, L-Ascorbate biosynthesis in higher plants: the role of VTC2, Trends Plant Sci. 13 (2008) 567-573.

[16] E. Ioannidi, M.S. Kalamaki, C. Engineer, I. Pateraki, D. Alexandrou, I. Mellidou, J. Giovannonni, A.K. Kanellis, Expression profiling of ascorbic acid-related genes during tomato fruits development and ripening and in response to stress conditions, J. Exp. Bot. 60 (2009) 663-678.

[17] M. Li, F. Ma, C. Guo, J. Liu, Ascorbic acid formation and profiling of genes expressed in its synthesis and recycling in apple leaves of different ages, Plant Physiol. Biochem. 48 (2010) 216-224.

[18] F. Nishikawa, M. Kato, H. Hyodo, Y. Ikoma, M. Sugiura, M. Yano, Ascorbate metabolism in harvested broccoli, J. Exp. Bot. 54 (2003) 2439-2448.

[19] V. Mittova, M. Volokita, M. Guy, M. Tal, Activities of SOD and the ascorbate-glutathione cycle enzymes in subcellular compartments in leaves and roots of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennelli*, Physiol. Plant. 110: (2000) 42-51.

[20] A. Saebo, T. Krekling, M. Appelgren, Light quality affects photosynthesis and leaf

anatomy of birch plantlets *in vitro*, Plant Cell Tiss. Org. 41 (1995)177-185.

[21] M.C. Wu, C.Y. Hou, C.M. Jiang, Y.T. Wang, C.Y. Wang, H.H. Chen, H.M. Chang, A novel approach of LED light radiation improves the antioxidant activity of pea seedlings, Food Chem. 101 (2007) 1753-1758.

[22] H.L. Xu, Q.C. Xu, Li F.L., Feng Y.Z., F.F. Qin, W. Fang, Applications of xerophytophysiology in plant production-LED blue light as a stimulus improved the tomato crop, Sci. Hortic. (Amsterdam) 148 (2012) 190-196.

[23] K.H. Lin, M.Y. Huang, W.D. Huang, M.H. Hsu, Z.W. Yang, C.M. Yang, The effects of red, blue, and white light-emitting diodes on the growth, development, and edible quality of hydroponically grown lettuce (*Lactuca sativa* L. var. *capitata*), Sci. Hortic. (Amsterdam) 150 (2013) 86-91.

[24] L.C. Zhang, G. Ma, M. Kato, K. Yamawaki, T. Takagi, Y. Kiriiwa, Y. Ikoma, H. Matsumoto, T. Yoshioka, H. Nesumi, Regulation of carotenoid accumulation and the expression of carotenoid metabolic genes in citrus juice sacs *in vitro*, J. Exp. Bot. 63 (2012) 871-886.

[25] C.G. Bartoli, J. Yu, F. Gómez, L. Fernández, L. McIntosh, C.H. Foyer, Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in Arabidopsis thaliana leaves, J. Exp. Bot. 57 (2006) 1621-1631.

[26] C.G. Bartoli, E.A. Tambussi, F. Diego, C.H. Foyer, Control of ascorbic acid synthesis and accumulation and glutathione by the incident light red/far red ratio in Phaseolus vulgaris leaves, FEBS Lett. 583 (2009)118-22.

[27] D.R. Gallie, The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth, J. Exp. Bot. 64 (2013) 433-443.

[28] L. Mastropasqua, G. Borraccino, L. Bianco, C. Paciolla, Light qualities and dose influence ascorbate pool size in detached oat leaves, Plant Sci. 183 (2012) 57-64.

[29] M.J. Li, F.W. Ma, M. Zhang, F. Pu, Distribution and metabolism of ascorbic acid in apple fruits (Malus domestica Borkh cv. Gala), Plant Sci.174(2008) 606-612.

[30] S.M. Bulley, M. Rassam, D. Hoser, W. Otto, N. Schünemann, M. Wright, E. MacRae, A. Gleave, W. Laing, Gene expression studies in kiwifruit and gene over-expression in Arabidopsis indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis, J. Exp. Bot. 60 (2009) 765-778.

[31] X.Y. Yang, J.X. Xie, F.F. Wang, J. Zhong, Y.Z. Liu, G.H. Li, S.A. Peng, Comparison of ascorbate metabolism in fruits of two citrus species with obvious difference in ascorbate content in pulp, J. Plant Physiol. 168 (2011) 2196-2205.

[32] E. Alós, M.J. Rodrigo, L. Zacarías, Differential transcriptional regulation of L-ascorbic acid content in peel and pulp of citrus fruits during development and maturation, Planta 239 (2014)1113-1128.

[33] F. Nishikawa, M. Kato, T. Kamo, R. Wang, H. Hyodo, Y. Ikoma, M. Sugiura, M. Yano, Enzymatic catabolism of ascorbate in florets of harvested broccoli during senescence, J. Jpn. Soc. Hortic. Sci. 70 (2001) 709-715.

[34] M. Kato, Y. Hayakawa, H. Hyodo, Y. Ikoma, M. Yano, Wound-induced ethylene synthesis and expression and formation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase, phenylalanine ammonia-lyase, and peroxidase in wounded mesocarp tissue of *Cucurbita maxima*, Plant Cell Physiol., 41 (2000) 440-447.

[35] I. Pateraki, M. Sanmartin, M.S. Kalamaki, D. Gerasopoulos, A.K. Kanellis, Molecular characterization and expression studies during melon fruit development and ripening of L-galactono-1,4-lactone dehydrogenase, J. Exp. Bot. 55 (2004) 1623-1633.

[36] M.L. Gomez, F.M. Lajolo, Ascorbic acid metabolism in fruits: activity of enzymes involved in synthesis and degradation during ripening in mango and guava, J. Sci. Food Agr. 88 (2008) 756-762.

[37] E. Cruz-Rus, M.A. Botella, V. Valpuesta, M.C., Gomez-Jimenez, Analysis of genes involved in L-ascorbic acid biosynthesis during growth and ripening of grape berries, J. Plant Physiol. 167 (2010) 739-748.

[38] T. Imai, Y. Ban, S. Terakami, T. Yamamoto, T. Moriguchi, L-Ascorbate biosynthesis in peach: cloning of six L-galactose pathway-related genes and their expression during peach fruit development, Physiol. Plant. 136 (2009) 139-149.

[39] F. Nishikawa, M. Kato, R. Wang, H. Hyodo, Y. Ikoma, M. Sugiura, M. Yano, Two ascorbate peroxidases from broccoli: identification, expression and characterization of their recombinant proteins, Postharvest Biol. Technol. 27 (2003) 147-156.

[40] T. Tokunaga, K. Miyahara, K. Tabata, M. Esaka, Generation and properties of ascorbic acid-overproducing transgenic tobacco cells expressing sense RNA for L-galactono-1,4-lactone dehydrogenase, Planta 220 (2005) 854-863.

[41] W.R. Briggs, C.F. Beck, A.R. Cashmore, et al, The phototropin family of photoreceptors, Plant Cell 13 (2001) 993-997.

[42] G. Ma, L.C. Zhang, M. Kato, Yamawaki K., Kiriiwa Y., Yahata M., Ikoma Y., Matsumoto H., Effect of blue and red LED light irradiation on β-cryptoxanthin accumulation in the flavedo of citrus fruits, J. Agric. Food Chem. 60 (2012) 197-201.

[43] R. Alba, M.M. Cordonnier-Pratt, L.H. Pratt, Fruit-localized phytochromes regulate lycopene accumulation independently of ethylene production in tomato, Plant Physiol. 123 (2000) 363-370.

[44] A. Schofield, G. Paliyath, Modulation of carotenoid biosynthesis during tomato fruit ripening through phytochrome regulation of phytoene synthase activity, Plant Physiol. Biochem. 43 (2005) 1052-1060.

[45] L.H Liu., D. Zabaras, L.E. Bennett, P. Aguas, B.W. Woonton, Effects of UV-C, red light and sun light on the carotenoid content and physical qualities of tomatoes during post-harvest storage, Food Chem. 115 (2009) 495-500.

[46] Food and Drug Administration, Kinetics of microbial inactivation for alternative food processing technologies: pulsed light technology, 2000.

[47] G. Oms-Oliu, I.Aguiló-Aguayo, O. Martín-Belloso, R. Soliva-Fortuny, Effects of pulsed light treatments on quality and antioxidant properties of fresh-cut

mushrooms (*Agaricus bisporus*), Postharvest Biol. Technol. 56 (2010) 216-222.

[48] F. Charles, V. Vidal, F. Olive, H. Filgueiras, H. Sallanon, Pulsed light treatment as new method to maintain physical and nutritional quality of fresh-cut mangoes, Innov. Food Sci. Emerg. 18 (2013) 190-195.

[49] S. Gatzek, G.L. Wheeler, N. Smirnoff, Antisense suppression of L-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated L-galactose synthesis, Plant J. 30 (2002) 541-553.

[50] P. Müller-Moulé, An expression analysis of the ascorbate biosynthesis enzyme VTC2, Plant Mol. Biol. 68 (2008) 31-41.

[51] S. Karpinski, C. Escorbar, B. Karpinska, G. Creissen, P.M. Mullineaux, Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress, Plant Cell 9 (1997) 627-640.

[52] M.J. Fryer, L. Ball, K. Oxborough, S. Karpinski, P.M. Mullineaux, N.R. Baker, Control of Ascorbate Peroxidase 2 expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of Arabidopsis leaves, Plant J. 33 (2003) 691-705.

[53] A.E. Eltayeb, N. Kawano, G.H. Badawi, H. Kaminaka, T. Sanekata, I. Morishima, T. Shibahara, S. Inanaga, K. Tanaka, Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone salt and polyethylene glycol stresses, Planta 225 (2007)1225-1264.

[54] L. Marty, W. Siala, M. Schwarzländer, M.D. Fricker, M. Wirtza, L.J. Sweetlove, Y. Meyer, A.J. Meyer, J.P. Reichheld, R. Hell, The NADPH-dependent thioredoxin system constitutes a functional backup for cytosolic glutathione reductase in Arabidopsis, Proc. Nati. Acad. Sci. USA 106 (2009) 9109-9114.

[55] A. Mhamdi, J. Hager, S. Chaouch, G. Queval, Y. Han, L. Taconnat, P. Saindrenan, H. Gouia, E. Issakidis-Bourguet, J.P. Renou, G. Noctor, Arabidopsis

GLUTATHIONE REDUCTASE1 plays a crucial role in leaf responses to intracellular hydrogen peroxide and in ensuring appropriate gene expression through both salicylic acid and jasmonic acid signaling pathways, Plant Physiol. 153 (2010) 1144-1160.

Table 1. Primers used for RT-PCR and cDNA lengths of genes related to ascorbic acid metabolism.

cDNA (Accession No.)	Sense primers (upper) and antisense primers (lower)	Length (bp)
CitVTC1 (HQ224946)	GGCTCTGGCTAGAGACAAATTGATTGACG CACTCTCATGCACCAAGACATTGCCTACA	500
CitVTC2 (HQ224948)	CTTGCCACAGAGGATTGACCGTGATAGCT CACTTGGGTGTCCAGAAGCTCTGAACTCA	453
CitVTC4 (HQ224949)	GTCGGGGTTGTCTACAATCCAATAATGG CAAGTCCTCCAGCTTCTTCAACAATCAC	340
CitGLDH (AB920387)	TCCAATCCCTTCATCACCGCCTCCTCTTCA CAAGACCCCCAAGACCACAACGCGCTAGA	777
CitAPX1 (AB920381)	ACCCCACTGTTAGCGAGGATTACAAGAAG GCACACTGTTGTTGGCATCATTCCAACCA	788
CitAPX2 (AB920382)	TCAGAGGACTCATTGCTGAGAAACACTGT AGCCACAATAAAGTACATGGATAGCCGCA	740
CitAPX3 (AB920383)	GAGCTCCGATGGCTTTACCGGTCGTTGAC TAAGAACTTGGCAGCGCTTGGGGCTGGCG	972
CitchAPX (AB920384)	ATTCTACAAAATCTGGAGGCCCTAATGGA ATTGGCGGAATCATTGGCACTTCCGCTCT	716
CitAO (AB920380)	CAGGCTCAGGATAGCTAGTACCACTGCA GAAATGGGGCTCAATATGGCAGTGAAATGC	898
CitMDAR1 (AB920389)	TAATGGCGAGCGTATCAAACTCGCTATCAT TATAACCACCACCAACAACAACCACCTTCT	660
CitMDAR2 (AB920390)	GATCGATCAATGGCGGGAAGAGCTTCA TCAACATGCTCAACTCTTCTCATTTCCCG	956
CitDHAR (AB920386)	ACACTCTCTCTGATCACTCAATTCCGCA CTCCAAGTCTCACACATACATTACTCGCA	745
CitGR (AB920388)	GTGGAACCTGTGTCATTCGTGGTTGTGTA GAAGGATGTATTCCCACCGTGCTGTCAAAT	1213
CitchGR (AB920385)	AGCGCTGTTCCATCTGCAGTCTTTTCTCA TCTACTCTACTAAACTCCTGCTGCAGCCT	426

Table 2. TaqMan MGB probes and primer sequences used for real-time quantitative RT-PCR of genes related to ascorbic acid metabolism.

cDNA	TaqMan MGB probe	Primers sequence	Orientation
CitVTC1	CCAGGTTTCTGGATGGA	GAAAGCTCTTCGCGATGGTT	Sense
		TAATCCCTCGGCTGTCCAAT	Antisense
CitVTC2	TGGAAACTCTCTGGAAGAC	TCAGAGGCCTCGTCTTTGAAG	Sense
		GCATCCGAGACTGTGTTGGA	Antisense
CitVTC4	CTCCTGTGCATTGAAT	GGTGAGATCCCTTCGGATGA	Sense
		CCGCATGCAATTCCACAA	Antisense
CitGLDH	CCTTCCCATTCTCCGAAG	GGCGCCGCCACATACTAT	Sense
		GATCTGGGCCTTCTTGTGCTT	Antisense
CitAPX1	CCGCCCTCTTGTTG	CCCTTCTGGATGACCCTGTTT	Sense
		TCCTCATCCGCAGCATATTTC	Antisense
CitAPX2	TCTGATCCGCCCCC	ACCCTGGAAGACCGGACAA	Sense
		GCATTTGGCAAGCGACCTT	Antisense
CitAPX3	ACGAAGACTGGTGGACC	GCGGGAACATCTGACGTGAA	Sense
		CTTCGTTCCTTATCGAGCCATT	Antisense
CitchAPX	AGGATGGCATGATGC	CATTTTGCCATCCTATTTTGGTT	Sense
		CCACTCCTCAATGTTCTTGTCATAA	Antisense
CitAO	CCGTCGGCTCACC	CAAGCCTCCCACAAATTTCC	Sense
		CGTTAATGGTGTTTTGAGTGTTAAGAA	Antisense
CitMDAR1	CTCTAAGGAGGCTTATGC	TGATGGACGCCTCTGTATCG	Sense
		AAGCCGGACGCTCATAAGG	Antisense
CitMDAR2	TCTGTGTTGGAAGTGGTG	GCTAGACTTCCCGGTTTCCAT	Sense
		GTACCACTCAGGAAGCAGTCTCTCT	Antisense
CitDHAR	CTTCAGGTAGCTCTTG	TTGGCACCAAAGCTGTACCA	Sense
		GGACAGTCCACTGCTTGAAATG	Antisense
CitGR	CAGAAATCTGGAAGGGAC	TGAAATGCGGGCAGTAGTTG	Sense
		CCTCGGGTGCAAATTAATTCC	Antisense
CitchGR	AGCCCTCCGTCTGAG	CGCCTACTCGGAAGATCAGAA	Sense
		CCTTAACCTCAGGACCAGTCATTC	Antisense

Figure legends

Fig. 1. The main AsA metabolism pathway in plants. VTC1, GDP-D-mannose pyrophosphorylase; VTC2, GDP-L-galactose phosphorylase; VTC4, L-galactose-1-P phosphatase; GLDH, L-galactono-1,4-lactone dehydrogenase; AsA, ascorbic acid; APX, ascorbate peroxidase; AO, ascorbate oxidase; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione.

Fig. 2. Changes in AsA contents in Satsuma mandarin, Valencia orange, and Lisbon lemon juice sacs during the ripening process. The results shown are the mean ± SE for triplicate samples. A, August; S, September; O, October; N, November; D, December; J, January; F, February.

Fig. 3. Changes in the expression of AsA metabolic genes in Satsuma mandarin, Valencia orange, and Lisbon lemon juice sacs during the ripening process. A, August; S, September; O, October; N, November; D, December; J, January; F, February. 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 genes under identical conditions. TaqMan MGB probes and sets of primers used for analyses are shown in Table 2. The results shown are the mean ± SE for triplicate samples.

Fig. 4. Effects of blue and red LED lights on AsA metabolism in juice sacs of Satsuma mandarin (a), Valencia orange (b), and Lisbon lemon (c) *in vitro*. The results shown are the mean ± SE for triplicate samples. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Tukey's HSD test.

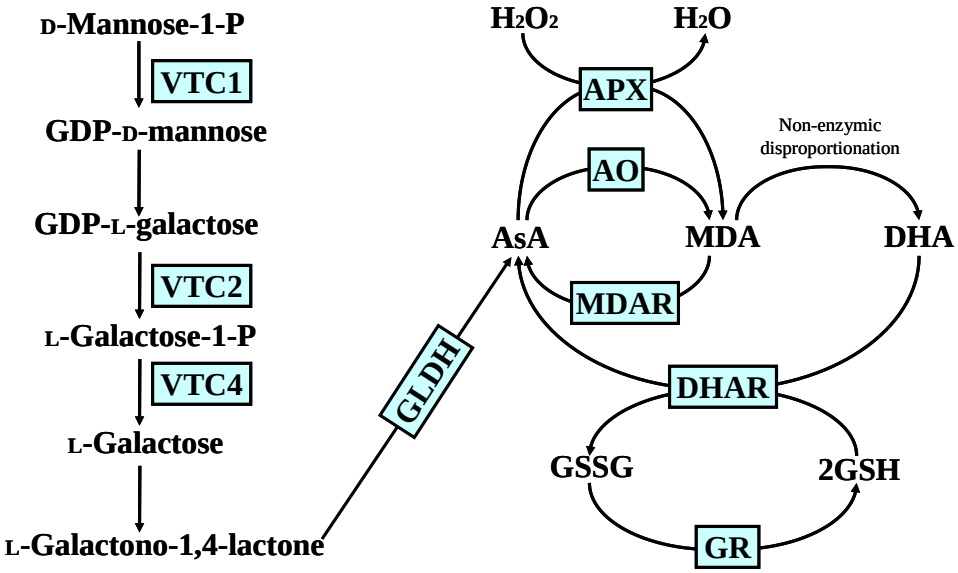
Fig. 5. Effects of different intensities of blue LED light on AsA metabolism in juice sacs of Satsuma mandarin (a), Valencia orange (b), and Lisbon lemon (c) *in vitro*. The results shown are the mean ± SE for triplicate samples. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Tukey's HSD test.

Fig. 6. Effects of continuous and pulsed irradiation of blue LED light on AsA

metabolism in juice sacs of Satsuma mandarin (a), Valencia orange (b), and Lisbon lemon (c) *in vitro*. The results shown are the mean ± SE for triplicate samples. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Tukey's HSD test.

Fig. 7. Effects of blue LED light on the expression of genes related to AsA metabolism in juice sacs of Satsuma mandarin (a), Valencia orange (b), and Lisbon lemon (c). 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 genes under identical conditions. TaqMan MGB probes and sets of primers used for analyses are shown in Table 2. The results shown are the mean ± SE for triplicate samples. Transcript expression levels were compared between the control and blue LED light at the corresponding time points by the Student's t-test; asterisks indicate significant differences, $*P < 0.05$; $** P < 0.01$.

Fig. 1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VTC1
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GDP-L-galactose
VTC2
L-Galactose-1-P
VTC4
L-Galactose
L-Galactono-1,4-lactone
GLDH
H2O2
H2O
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disproportionation
AsA
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DHA
MDAR
DHAR
GSSG
2GSH
GR

Fig. 2

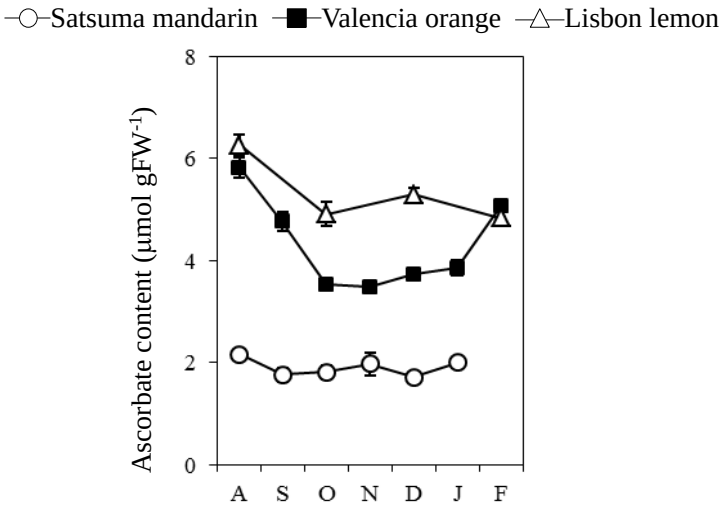
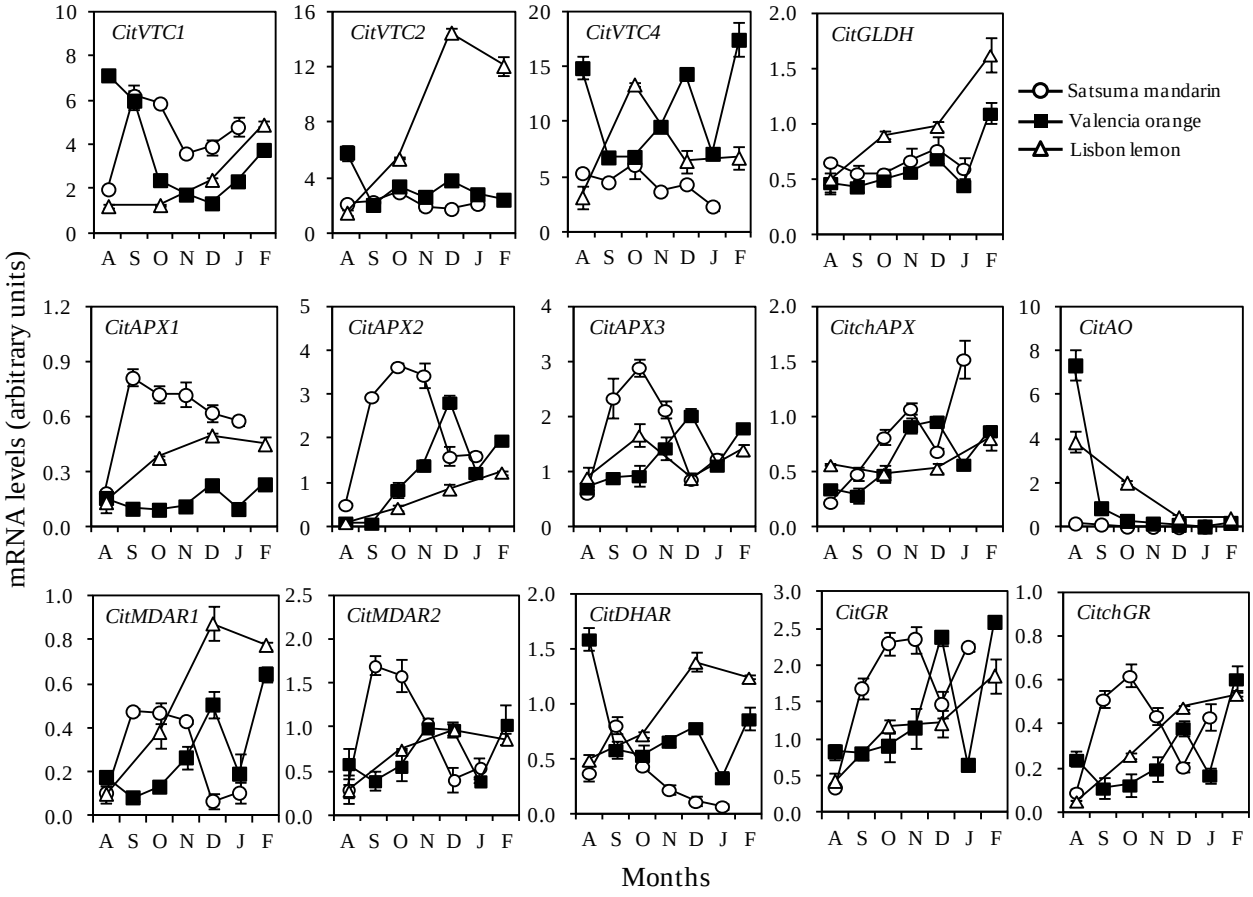

Satsuma mandarin
Valencia orange
Lisbon lemon
Ascorbate content (μmol gFW-1)
8
6
4
2
0
A
S
O
N
D
J
F

Fig. 3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CitAO
CitMDAR1
CitMDAR2
CitDHAR
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CitchGR
Satsuma mandarin
Valencia orange
Lisbon lemon
mRNA levels (arbitrary units)
A S O N D J F
Months

Fig. 4

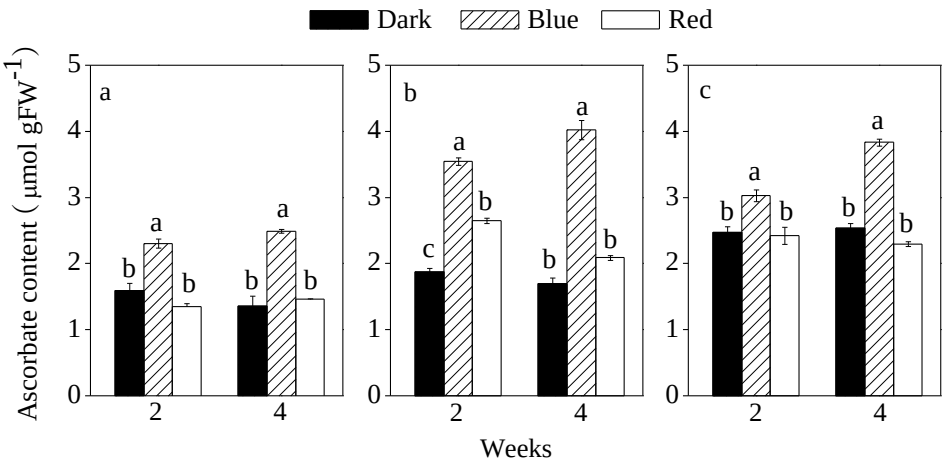


Fig. 5

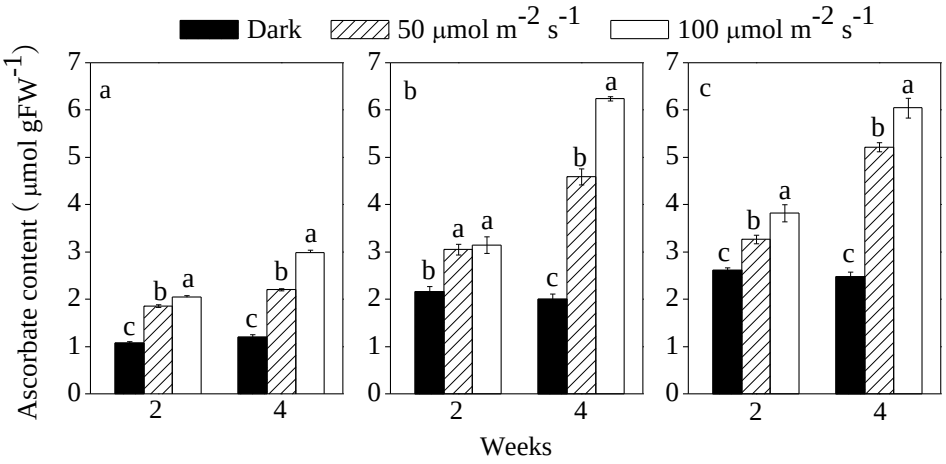

Dark
50 μmol m-2 s-1
100 μmol m-2 s-1
Ascorbate content (μmol gFW-1)
Weeks
a
b
c
0
1
2
3
4
5
6
7
2
4

Fig. 6

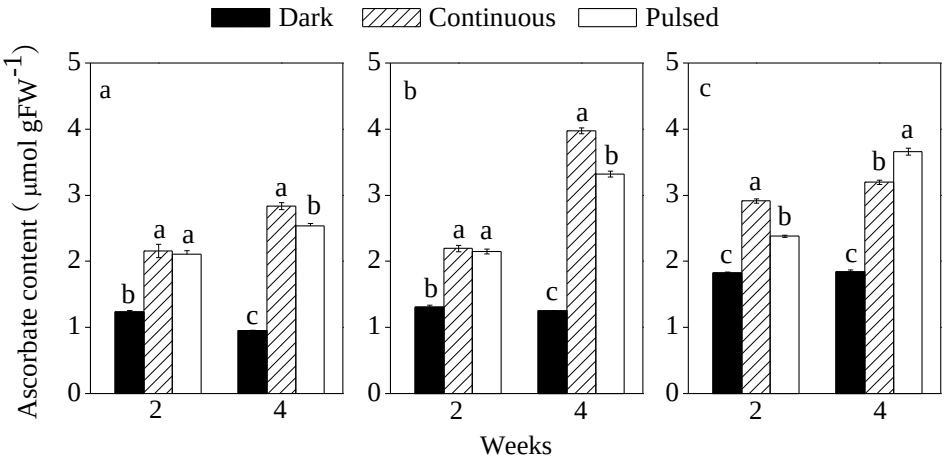
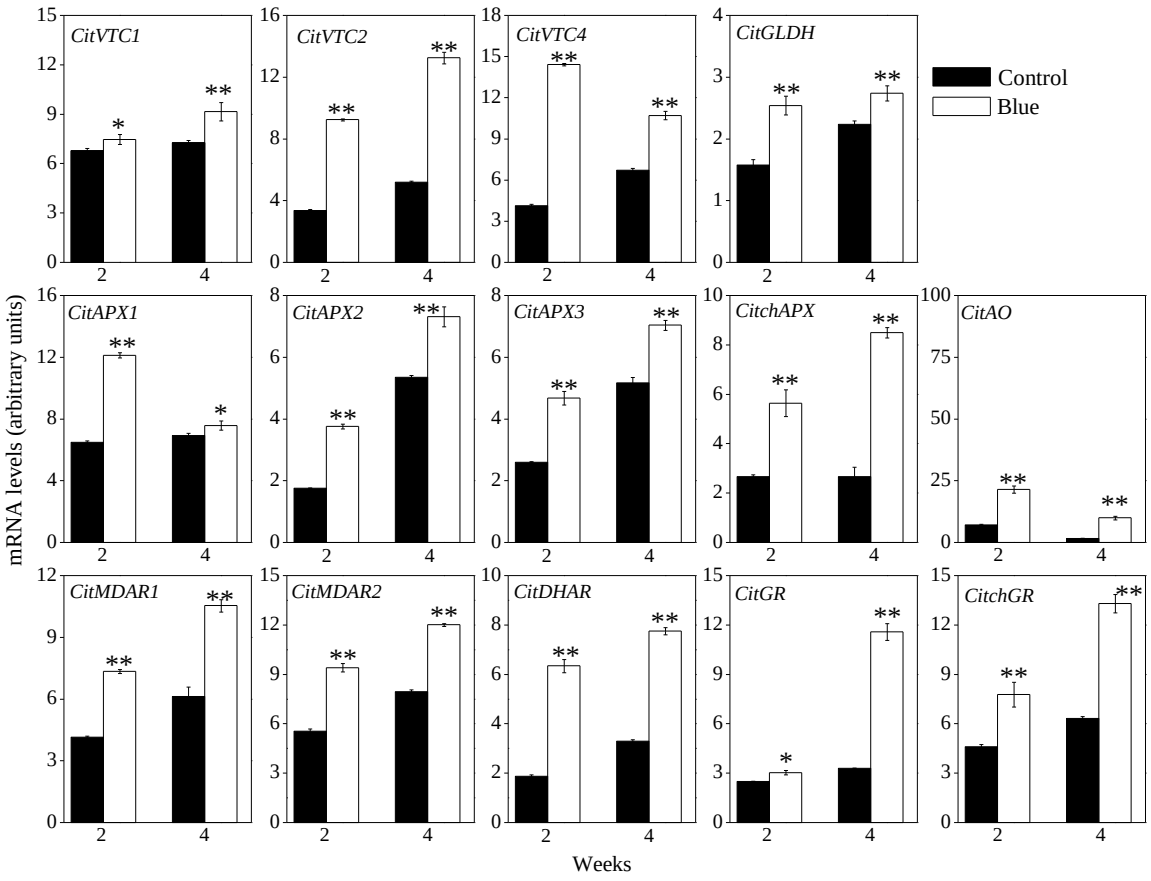

Dark
Continuous
Pulsed
Ascorbate content (μmol gFW-1)
Weeks
a
b
c
0
1
2
3
4
5
2
4

Fig. 7

a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CitDHAR
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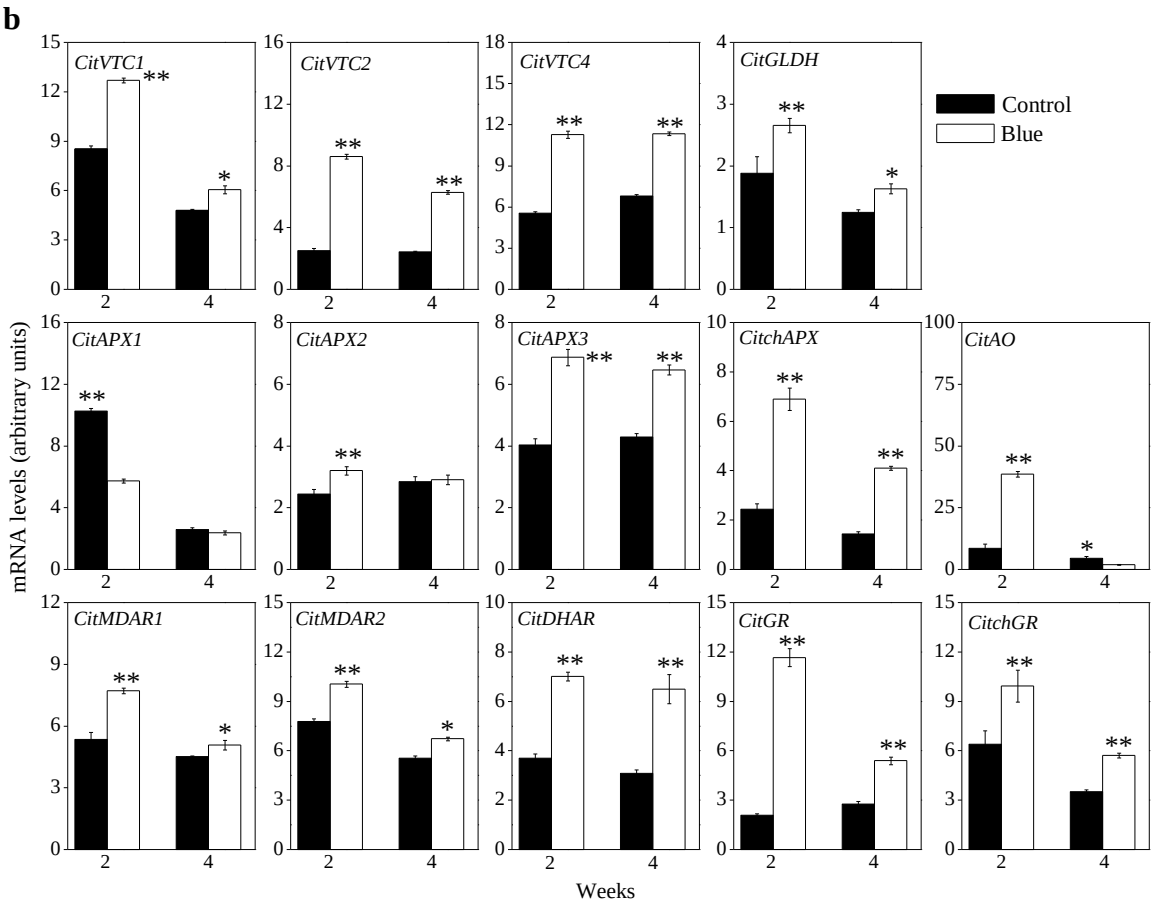
CitchGR
Control
Blue
mRNA levels (arbitrary units)
Weeks

Fig. 7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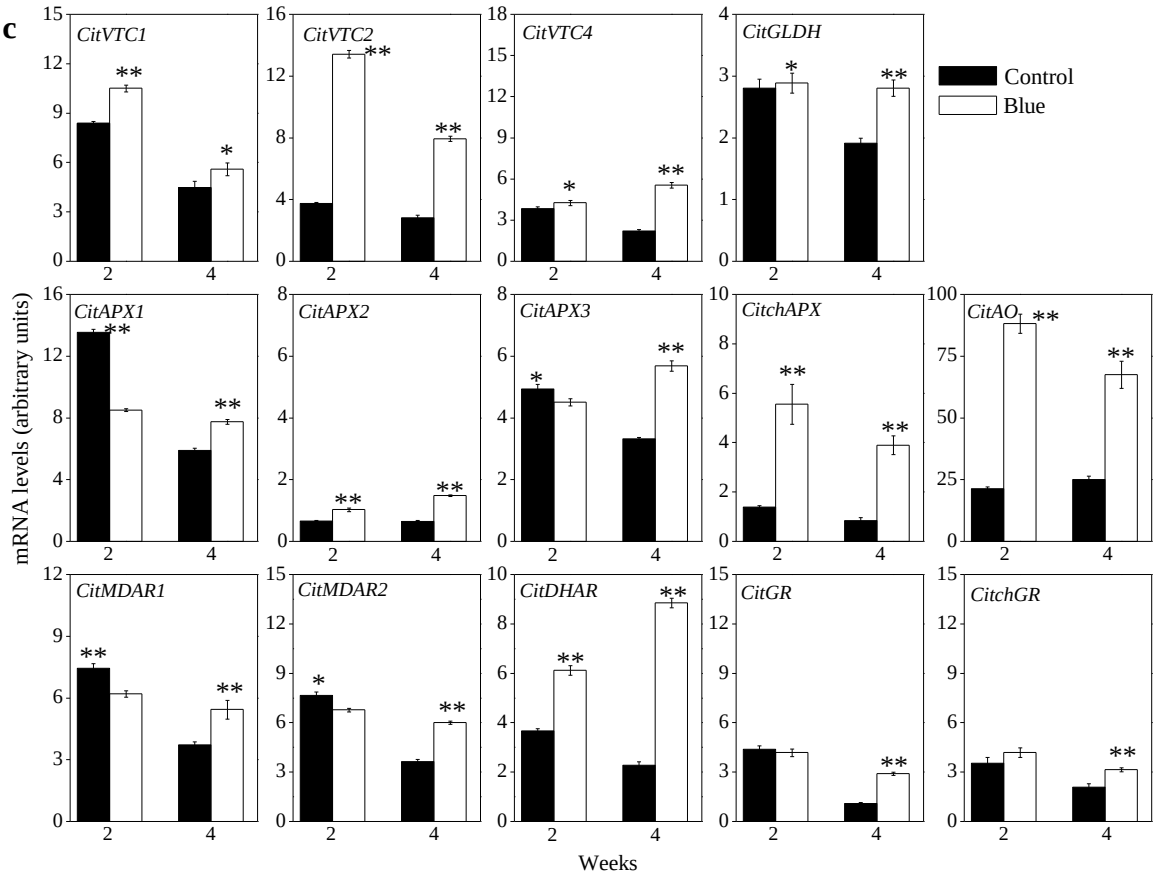
mRNA levels (arbitrary units)
Weeks

Fig. 7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CitVTC1
CitVTC2
CitVTC4
CitGLDH
CitAPX1
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CitAPX3
CitchAPX
CitAO
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CitDHAR
CitGR
CitchGR
Control
Blue
mRNA levels (arbitrary units)
Weeks