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Abstract

In the present study, we studied the effects of gibberellic acid (GA) on chlorophyll and carotenoid metabolites and related gene expression during the regreening process in Valencia orange fruits (*Citrus sinensis* Osbeck). During the regreening, fruits treated with GA turned green much faster than those of the control. Compared with untreated fruits, chlorophyll accumulation was induced and the content of carotenoids (β-cryptoxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin) was decreased by the GA treatment. Chlorophyll and carotenoid contents following GA treatment appeared to be highly regulated at the gene transcription level. Correspondingly, the up-regulation of chlorophyll biosynthesis genes (*CitGGDR*, *CitCHL27*, *CitPORA*, and *CitCAO*) and down-regulation of degradation genes (*CitCLH1*, *CitSGR*, *CitPPH*, *CitPAO*, and *CitRCCR*) led to the increase of chlorophyll contents, and the down-regulation of carotenoid biosynthesis genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, and *CitHYb*) led to the decrease of carotenoid contents. These observations indicated that GA acted as a crucial regulator in the regreening process of citrus fruits.

Keywords: Carotenoid, Chlorophyll, Gibberellic acid, Regreening, Valencia oranges

1. Introduction

Citrus fruits accumulate a diversity of pigments at different mature stages. In general, the color of citrus fruits is determined by different classes of pigments, namely chlorophylls, carotenoids, and anthocyanins. In the immature fruit, the fruit flavedos is abundant in chlorophylls during the summer season in response to high temperatures of air and soil. The fruit development generally occurs in mid-autumn when the temperature goes down. During fruit maturation, the color development in the flavedos is associated with the accumulation of carotenoids and the simultaneous degradation of chlorophylls (Kato et al., 2004; Rodrigo et al., 2013).

In plants, geranylgeranyl diphosphate (GGPP) is a precursor for the synthesis of chlorophylls. Chlorophyllide (a) is synthesized from glutamate in plastids, and the phytol side chain derives from GGPP that is produced via isopentenyl diphosphate (IPP) in the plastidial methylerthritol 4-phosphate (MEP) pathway. GGPP is then reduced to phytylPP by geranylgeranyl-PP reductase (GGDR). The condensation of those molecules to form chlorophyll *a* (Chl a) is catalyzed by chlorophyll synthase (CS). Chl a is the major component of chlorophylls in citrus fruit flavedos. Subsequently, Chl a can be converted into chlorophyll *b* (Chl b) by chlide a oxygenase (CAO) (Joyard et al., 2009; Rodrigo et al., 2013; Ma et al., 2021a, b). The four basic steps of chlorophyll degradation start with the conversion of Chl b to Chl a by Chl b reductase (CBR) and 7-hydroxymethyl Chl a reductase (HCAR), then the Chl a is converted to chlorophyllide a by chlorophyllase (Chlase) to remove of the side chain attached to the tetrapyrrole macrocycle. Afterwards, the magnesium in the center of chlorophyllide is removed by action of magnesium dechelatase (STAY-GREEN, SGR), thereby producing pheophytin, which is then catabolized to pheophorbide and free phytol by action of pheophorbide hydrolase (PPH). Finally, pheophorbide a is converted to the “red chlorophyll catabolite” (RCC) by action of pheophorbide a oxygenase (PAO), followed by conversion of RCC into “fluorescent chlorophyll catabolites” (FCCs) by red chlorophyll catabolite reductase (RCCR) (Hörtensteiner, 1999; Joyard et al., 2009; Rodrigo et al., 2013; Xie et al., 2019; Wang and Grimm, 2021).

In parallel, the synthesis of carotenoids starts from the condensation of two molecules of GGPP to ultimately form phytoene by action of phytoene synthase (PSY). Then phytoene is converted to lycopene by phytoene dehydrogenase (PDS) and ξ-carotene desaturase (ZDS). Lycopene molecules are

standing at a branching point leading either to α-carotene or to β-carotene, depending on two different cyclases, lycopene β-cyclase (LCYb) and lycopene ε-cyclase (LCYe). α-Carotene is converted to lutein catalyzed by ε-ring hydroxylase (HYe) and β-ring hydroxylase (HYb). β-Carotene is converted to β-cryptoxanthin and zeaxanthin via a two-step hydroxylation by HYb. In addition, the conversion from zeaxanthin to violaxanthin is catalyzed by zeaxanthin epoxidase (ZEP) (Kato, 2012; Rodrigo et al., 2013; Xie et al., 2019).

In some citrus cultivars, when the fruit is left on the tree till the spring or summer season, the color of the fruits will reverse from orange to green, and this process is called “regreening” of the citrus fruits. Previous studies found that the regreening was depending on environmental conditions during the summer season (Caprio, 1956). The conversion from chromoplasts to chloroplasts occurred in the peel, which was correlated with the decrease in carotenoids and the gradual accumulation of chlorophyll during fruit regreening (Thomson et al., 1967; El-zeftawi, 1977). The regreening fruits contained higher levels of chlorophylls in the stem end area than in the apical and equatorial area of the peel (Coggins and Lewis, 1962). In previous studies, Rasmussen (1973) and El-zeftawi and Garrett (1978) demonstrated that the regreening in citrus was induced by exogenous gibberellic acid (GA). In another study, Farag et al. (2014) reported that the occurrence of regreening was not only correlated with a high accumulation of nitrogen, but also with the temperature of the branches and growth degree of Valencia oranges. High temperature of the branches induced the uptake of nitrogen, and promoted the regreening in the fruits. In addition, light can stimulate the regreening process in citrus fruits (Saks et al., 1988). Ma et al. (2021a) reported that blue LED light irradiation stimulated regreening by inducing chlorophyll accumulation and increasing the contents of all-*trans*-violaxanthin, β-carotene, and lutein, while decreasing the content of 9-*cis*-violaxanthin in the fruit flavedo *in vitro*. This was correlated with the expression of biosynthesis genes, such as the up-regulation of *CitLCYe* and down-regulation of *CitLCYb2*. Moreover, citrus varieties, location on the trees, rootstock, and the number of seeds per fruit also affected the regreening process in citrus fruits (El-Zeftawi, 1977; Huff, 1983). In a previous study, Hsu et al. (1989) found that application of the bioregulators [(*N*,*N*-diethylamino) ethoxy] benzophenone, (*N*,*N*-diethylamino) ethyl p-bromobenzoate, and *N*,*N*-diethyloctylamine before harvest

not only reduced the regreening by reducing chlorophyll biosynthesis but also increased the total xanthophyll content in the flavedo of Valencia oranges.

In previous studies, it was reported that treatment by exogenous GA induced regreening of citrus fruits on tree, but the effects of GA treatment on the expression of genes being associated with pigment accumulation during the regreening remained unknown. In the study here, we investigated the effects of GA on the accumulation of chlorophyll and carotenoid metabolites and on related gene expression during the regreening in Valencia oranges. The outcome of this research should help gaining a deeper insight into the role of phytohormones in regulating the pigment profiles during the regreening.

2. Materials and Methods

2.1. Plant Materials and Treatments

In this study, citrus fruits of 'Valencia orange' (*Citrus sinensis* Osbeck) were grown in the Fujieda farm of the Shizuoka University (Japan), harvested and used as materials. Fruits on the tree were separated into two parts, control (non-treated) and GA-treated group. In this study, we used GA_3 solution from Meiji Seika Pharma, Tokyo, Japan. On the tree, plastic sheets were used to separate and protect the control fruits when GA solution was sprayed. The fruits were sprayed with 500 μM GA on the tree every 2 weeks for 3 times from April 1st, 2020. After that, the fruits in each treatment were randomly harvested from the tree every 2 weeks. As to fruit sampling, the flavedos of fruit were separated into two parts; the part from stem end to the middle of fruit was classified as the top part, and the rest of fruit was classified as the bottom part. The flavedos in each part of the fruit were frozen immediately in liquid nitrogen, and then stored at -80 °C until analysis. The samples were used for chlorophyll, carotenoid, and related gene expression analyses.

2.2. Color Analysis

The peel color of fruits from each treatment was measured using a Nippon Denshoku NR-12A colorimeter at 3 positions on the equatorial plane of each part in top and bottom. The color changes were present by hue angle ($H°$) and citrus color index (CCI), which calculated from $H°$ = arctangent (b*/a*) and CCI = 1000×a*/(L*×b*) (Zhou et al., 2010; Ma et al., 2015; Xie et al., 2019).

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141 **2.3. Extraction and Analysis of Chlorophylls**

142 Chl a and Chl b were extracted from flavedos by using *N*,*N*-dimethylformamide and incubated
143 overnight at room temperature, followed by centrifugation at 3000 rpm for 10 min, and absorbance was
144 measured spectrometrically at 664 and 647 nm. The chlorophyll contents were calculated according to
145 Moron (1982) and expressed as milligrams per kilogram fresh weight. Chl a = $(12.64 \times OD_{664}) - (2.99$
146 $\times OD_{647})$; Chl b = $(-5.6 \times OD_{664}) + (23.26 \times OD_{647})$; Total chlorophyll = $(7.04 \times OD_{664}) + (20.27 \times$
147 $OD_{647})$.

148

149 **2.4. Extraction and Analysis of Carotenoids**

150 The contents of β-carotene, β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and
151 lutein in the flavedos were determined in three replications. About 0.5 g of the frozen sample was
152 homogenized with an extraction solution (hexane/acetone/ethanol, 2/1/1, v/v) containing 10 % (w/v)
153 basic magnesium carbonate, followed by centrifugation at 3000 rpm for 20 min. The organic solvents
154 were evaporated to dryness at 35 °C under vacuum condition. After this step, the samples were
155 saponified overnight with 12 mL diethylether containing 0.1 % (w/v) 2,6-di-*tert*-butyl-4-methylphenol
156 (BHT) and 8 mL 20 % (w/v) methanolic KOH. After saponification, the NaCl-saturated water was
157 added to remove water-soluble extracts. The samples were re-extracted with diethyl ether, which was
158 then removed by evaporation at 35 °C until dryness. Carotenoid residues were repeatedly dissolved in
159 5 mL of *tert*-butyl methyl ether (TBME)/methanol (1/1, v/v) containing 0.5 % (w/v) BHT and then kept
160 in amber vial under -20 °C. Carotenoids were analyzed by use of a reverse-phase HPLC system (Jasco,
161 Tokyo, Japan). The YMC Carotenoid S-5 column (Waters, Milford, MA, USA) was used. The
162 carotenoids contents were calculated using the calibration curves and expressed as milligrams per
163 kilogram fresh weight (Kato et al., 2004). The total carotenoids were calculated by summing β-carotene,
164 β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and lutein.

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166 **2.5. Extraction and qRT-PCR Analysis of Chlorophyll and Carotenoid Metabolisms**

The total RNA from flavedos was extracted by phenol-chloroform according to Kato et al. (2004). The RNeasy Mini Kit (Qiagen, Hilden, Germany) with DNase digestion was used for purified the total RNA. In order to synthesis cDNA, 2 μg of purified RNA were reverse transcribed using TaqMan Reverse Transcription Reagent (Applied Biosystems, Foster City, CA, USA) and random hexamer at 37 °C.

In this study, qRT-PCR was carried out in the three replications. TaqMan MGB probes and the set of primers for chlorophyll biosynthesis genes (*CitGGDR*, *CitCHLH*, *CitCHLM*, *CitCHL27*, *CitPORA*, *CitCAO*, and *CitCS*), chlorophyll degradation genes (*CitCLH1*, *CitCLH2*, *CitSGR*, *CitPPH*, *CitPAO*, and *CitRCCR*), and carotenoid biosynthesis genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, and *CitHYe*) were previously described by Kato et al. (2004, 2006); Ma et al. (2021a). The Real-Time PCR reaction mixture contained 900 nM of primers (forward and reverse primer), 250 nM of TaqMan MGB Probe. The RT-qPCR was carried out by using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The thermal cycling conditions of the cDNA template were as follows: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

2.6. Statistical Analysis

All values are shown as the mean ± standard error (SE). Statistical differences between control and GA treatment were evaluated with *t*-test at $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ levels.

3. Results

3.1. Changes of color in the flavedos of the GA-treated fruits

In order to explore the effect of GA treatment on the color change in flavedos of Valencia orange fruits on trees, 500 μM of GA was sprayed on tree every 2 weeks for 3 times, which started from April 1st, 2021. The non-treated fruits were used as control. Evidently, the color of the flavedos changed from dark orange to pale orange, and then turned gradually green during the regreening process. As shown in Figure. 2, visible changes in the flavedo color were observed in the control as well as the GA-treated fruits during the experiment period. In the control, the green color of flavedo appeared from the 8th week. Clearly, GA treatment accelerated the regreening process, which occurred from the 6th week.

Moreover, the regreening appeared earlier in the top part of the fruits than the bottom parts in both control and GA-treated fruit flavedos.

The color changes in the flavedos were determined by the hue angle and citrus color index (CCI). The hue angle from 180° to 0°#epresents the color changing from green to red, and the CCI from negative to positive values represents the color changing from green to orange (Zhou et al., 2010; Ma et al., 2015; Xie et al., 2019). In the study here, the color changes were separately measured in the top and bottom parts of the fruits. Evidently, with the color changing from orange to green, the value of the hue angle increased rapidly, while that of the CCI decreased slightly in the control and after GA treatment during the regreening process (Figure. 3). Compared with the control, the hue angle in GA-treated fruits was higher in both top and bottom parts throughout the experimental periods. In contrast, the CCI values in the GA treated fruits were significantly lower than in the control throughout the experimental periods. In addition, a faster change of color was observed in the top than in the bottom part of the fruits. The hue angles in the top parts were higher and the CCI values were lower than the bottom in the control and GA-treated fruits. These values were consistent with the visual color changes in the fruits during the regreening process (Figure. 2).

3.2. Changes of chlorophyll contents and the expression of chlorophyll biosynthesis and degradation genes in the flavedos of GA-treated fruits

The change of color in flavedos could be attributed to the differences in the accumulation of chlorophylls and carotenoids. During the regreening, the contents of Chl a, Chl b, and total chlorophyll in the top and bottom parts of the fruits were simultaneously increased by the GA treatment as compared with the control (Figure. 4). Moreover, the top part of the fruits in the GA treatment group showed significantly higher contents of Chl a, Chl b, and total chlorophyll than in the control at the 6^{th} and 8^{th} weeks. At the bottom of the fruits, the contents of Chl a and of total chlorophyll was significantly higher in the GA treatment group than in control fruits at the 6^{th} and 8^{th} week, and the content of Chl b in the GA group was significantly higher than in the control at the 6^{th} week.

Regarding to the expression of chlorophyll metabolic genes, 7 genes (*CitGGDR*, *CitCHLH*, *CitCHLM*, *CitCHL27*, *CitPORA*, *CitCS*, and *CitCAO*) involved in chlorophyll biosynthesis and 6 genes

(*CitCLH1*, *CitCLH2*, *CitSGR*, *CitPPH*, *CitPAO*, and *CitRCCR*) involved in chlorophyll degradation were investigated in this study. During regreening, the expression of *CitGGDR*, *CitCHL27*, *CitPORA*, and *CitCAO* tended to increase throughout the experimental period (Figure. 5). In the top part of fruits, the expression of *CitGGDR*, *CitPORA*, and *CitCAO* was significantly up-regulated by the GA treatment at the 4th and 6th weeks. In addition, the expression of *CitCHLM* and *CitCS* in the GA treatment was higher than the control in the top part at the 4th week. The expression of *CitCHL27* in the top part was significantly up-regulated by the GA treatment throughout the experimental period. In the bottom part of fruits, the expression of *CitPORA* in the GA treatment was significantly up-regulated at the 6th and 8th weeks. The expression of *CitCAO* in the bottom part was significantly up-regulated by the GA treatment at the 4th and 6th weeks. As shown in Figure. 6, the results showed that the expression of chlorophyll degradation related genes (*CitCLH1*, *CitSGR*, *CitPPH*, *CitPAO*, and *CitRCCR*) in the GA treatment was markedly lower than the control in both the top and bottom parts of fruits during the experiment period.

3.3. Changes of carotenoids contents and the expression of carotenoid biosynthesis genes in the flavedos of GA-treated fruits

In this study, the major carotenoids, including β-carotene, β-cryptoxanthin, lutein, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin, were detected in the flavedos. During regreening, the contents of those five major carotenoids and the total carotenoids decreased in the control and after GA treatment (Figure. 7). In the top part of fruits, the contents of β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and total carotenoid in the GA treatment decreased more rapidly than the control. In the top part of GA-treated fruits, the contents of all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and β-cryptoxanthin were significantly lower than the control. Similarly, in the bottom part of GA-treated fruits, the contents of β-carotene, β-cryptoxanthin, lutein, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin were significantly lower than the control throughout the experimental period.

In this study, the expression of 8 carotenoid biosynthesis genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, and *CitHYe*) was investigated. In the control and GA treatment, the expression levels of the carotenoid biosynthesis genes were high at the beginning, and

then decreased rapidly at the 4th week of the experiment period (Figure. 8). In the top and bottom parts of fruits, the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, and *CitHYb* was significantly down-regulated by the GA treatment, while the expression of *CitLCYe* was significantly up-regulated by the GA treatment compared with the control during the experiment period.

4. Discussion

In general, citrus fruits turn from green to yellow, orange, or red when they become mature in winter season. However, in several citrus varieties, such as Valencia oranges, the fruits will reverse from orange to green when left on the trees until spring or summer season (Caprio, 1956). In the past decades, it was reported that exogenous plant hormones, such as GA and benzyl adenine (BA), not only delayed the degreening, but also induced the regreening in flavedo of citrus fruits (Rasmussen, 1973). However, the effects of exogenous GA on the expression of genes associated with pigment accumulation were not explored. In the present study, in order to elucidate the molecular mechanism underlying the regreening induced by exogenous GA, the changes of carotenoid and chlorophyll contents and the expression of genes associated with carotenoid and chlorophyll biosynthesis were investigated in the flavedo of Valencia oranges. The results showed that hue angle value increased rapidly, while CCI values decreased slightly in the control and GA treatment group during the regreening process. Compared with the control, the flavedos treated with GA turned green faster, and showed higher hue angle and lower CCI values throughout the experimental period (Figure. 3). These observations suggest that exogenous GA induced the regreening in the flavedo of Valencia oranges. In addition, we found an interesting phenomenon that the regreening was more evident in the top part of fruits than the bottom part in both control and GA treatment, which indicated that the regreening process might start from the top part of the citrus fruits. In citrus fruits, it has been reported that the regreening was affected by several factors, such as light, temperature, nutrition, and plant hormones. The occurrence of regreening was stimulated by nature light in the flavedos. In addition, temperature was also a key factor for inducing regreening in citrus fruits. In citrus, the regreening of fruits occurs in the late spring and summer season. Iglesias et al., (2007) reported that the high temperature stimulated the up-take of nitrogen and formation of vegetative growth hormone for the new re-growth such as

279 flowering and fruit set, which might result in the fruits on tree regreening. In a previous study of our
280 group (Ma et al., 2021a), it was found that blue LED light effectively induced regreening in the flavedos
281 *in vitro*. In the *in vitro* experiment, we sampled the flavedos from the whole citrus fruits, and then
282 excised and placed them on the MS medium randomly. The environmental conditions, such as light,
283 water stress, and temperature, were identical in the *in vitro* culture system, and no difference in the
284 occurrence of regreening was observed between the top and bottom parts in response to blue LED light.
285 Therefore, we deduced that the phenomenon that regreening started from the top part might be attributed
286 to the different environmental conditions and nitrogen accumulation between the top and bottom parts
287 of the fruits on the tree.

288 In plants, the appearance of green color was associated with the accumulation of chlorophylls
289 during the regreening (Koiwa et al., 1986; Preberg et al., 2008; Chen et al., 2012). In previous studies,
290 it was found that the regreening is accompanied with the reversion of chromoplasts to chloroplasts and
291 the formation of new chloroplasts (Mackinney, 1961). The reversion of chromoplasts to chloroplasts
292 occurred with the disappearance or reduction in size and number of plastoglobules and the formation of
293 new thylakoids or reformation of thylakoids, leading to normal chloroplast structure and photosynthetic
294 activity (Thomson et al., 1967; Preberg et al., 2008; Devidé and Ljubešić, 1974; Egea et al., 2010). In
295 the present study, the results showed GA treatment induced the accumulation of chlorophylls in the
296 flavedo of Valencia oranges. During regreening, the chlorophyll *a*, *b* and total chlorophyll contents were
297 increased by the GA treatment throughout the experimental period, and their contents in the top part
298 were significantly higher than those in the bottom part of the fruits. In citrus fruits, the accumulation of
299 chlorophylls was closely related to the expression levels of genes involved in chlorophyll metabolism.
300 Geranylgeranyl reductase (GGDR) is a key enzyme of chlorophyll biosynthesis catalyzing the
301 conversion of GGPP to phytyl-PP (Figure. 1), which is then followed by the condensation the phytol
302 chain with the porphyrin ring that is synthesized from glutamate in chloroplasts (Ma et al., 2021b). The
303 decrease in the gene expression of the *GGDR* was observed during fruit ripening, thereby explaining
304 the decrease in chlorophyll accumulation (Alós et al. 2006). Several studies reported that exogenous
305 GA was commonly used to delay senescence and loss of chlorophyll in various citrus fruits during pre-
306 harvest or post-harvest (Porat et al., 2001; Alós et al., 2006; Gambetta et al., 2014). It delayed

degreening by upregulating the transcription of magnesium chelatase in the citrus fruits (Fujii et al., 2008) . In addition, the expression of the gene encoding chlorophyll-degrading pheophorbide a oxygenase (PAO) was repressed in citrus fruits treated with exogenous GA during fruit degreening (Alós et al., 2006). Li et al. (2010) found that the expression of genes encoding chlorophyll catabolism enzymes including Chlase and magnesium dechelatase was down-regulated by GA in the senescing shoots of *Paris polyphylla* var. *yunnanensis* (Franch.). In the present study, we found that the expression level of *CitGGDR* was higher in the top part of GA-treated fruits as compared with the control, while the bottom part was not affected throughout the experimental period. This indicated that chlorophyll synthesis was activated, resulting in the regreening process to occur earlier in the top part than in the bottom part of fruit flavedos. In addition, *CitCHL27* and *CitPORA* were highly expressed in the top part of GA-treated fruits as compared with the control. The expression of *CitCAO*, which was involved in Chl b synthesis, was significantly up-regulated by the GA treatment during the regreening process. Furthermore, the expression of chlorophyll degradation genes (*CitCLH1*, *CitSGR*, *CitPPH*, *CitPAO*, and *CitRCCR*) was low in the GA-treated fruits during the regreening process. Thus, these results suggested that the exogenous GA induced the accumulation of chlorophylls by enhancing the expression of chlorophyll biosynthesis genes and repressing the expression of chlorophyll degradation genes in the flavedos during the regreening process. The results from the previous studies and our study as well convey that the application of GA not only delayed the degreening but also induced some regreening in citrus fruits.

In citrus, carotenoids are massively accumulated in the mature fruits, which are the important pigments responsible for the bright red, yellow, and orange colors in the flavedo of citrus fruits. In the green stage, the fruit flavedos accumulated high levels of β,ε-carotenoids (α-carotene and lutein), which were characteristic chloroplast carotenoids. When the fruits turned from green to orange, the contents of β,ε-carotenoids decreased, and an accumulation of β,β-carotenoids (β-cryptoxanthin, all-*trans*-violaxanthin and 9-*cis*-violaxanthin) was observed, which were characteristic chromoplast carotenoids of citrus fruits (Kato et al., 2004; Ma et al., 2016). The change in carotenoid composition was regulated by the expression of genes involved in carotenoid biosynthesis. During fruit ripening, the accumulation of β,β-xanthophylls in the flavedos can be explained by the increase in transcription levels of *CitPSY*,

CitPDS, *CitZDS*, *CitLCYb*, and *CitHYb* genes in the flavedo of citrus fruits. In parallel, the contents of β,ε-carotenoids (mainly lutein) decreased along with the down-regulation of *CitLCYe* during fruit ripening (Kato et al., 2004; Rodrigo et al., 2004; Alquézar et al., 2008). In citrus fruits, GA was applied to delay the degreening during fruit ripening, by down-regulating almost all carotenoid biosynthesis genes, especially the expression of *CitPSY*, *CitHYb*, and carotenoid cleavage dioxygenases in flavedo of citrus fruits (Fujii et al., 2008 and Ma et al., 2021a). In addition, GA treatment not only repressed the expression of carotenoid genes in flavedos but also in the juice sacs. Zhang et al. (2012) reported that the accumulation of carotenoids was significantly decreased and the expression of genes related to carotenoid metabolism (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2* , *CitHYb*, *CitZEP*, *CitNCED2* and *CitNCDE3*) was repressed in the juice sacs of Valencia oranges treated with GA *in vitro*. In the present study, the results showed that the content of total carotenoid significantly decreased in the control and GA treatment during the regreening process, which was well consistent with previous study of El-Zeftawi and Garrett (1978). Compared with the control, the contents of β,β-xanthophylls, including all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and β-cryptoxanthin, were decreased by the GA treatment. In our study here, it was shown that the expression of carotenoid biosynthesis genes significantly decreased during the regreening process. In the GA treatment, the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, and *CitHYb* genes was down-regulated, which led to the reduction of β,β-xanthophylls contents in the flavedos. These results suggested that GA treatment prevented the accumulation of carotenoid by suppressing the expression of biosynthesis genes, which led to the color of the flavedos turned from dark orange to pale orange during the regreening process.

5. Conclusion

In the present study, the results showed that the regreening was induced by the GA treatment, and it was more obvious in the top part of the fruits than the bottom part. Evidently, the regreening process started from the top part of citrus fruits. The GA treatment induced the accumulation of chlorophyll *a* and *b*, and decreased contents of β-cryptoxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin compared with the control. With regard to the expression of genes involved in chlorophyll metabolism, the up-regulation of chlorophyll biosynthesis genes (*CitGGDR*, *CitCHL27*, *CitPORA*, and

CitCAO) and down-regulation of degradation genes (*CitCLH1* , *CitSGR*, *CitPPH*, *CitPAO*, and *CitRCCR*) led to the increase of chlorophyll contents in GA-treated fruits. In addition, the down-regulation of the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2* , and *CitHYb* led to a reduction of carotenoid contents in the GA treatment group. This study provided deeper knowledge on the roles of GA in regulating of chlorophyll and carotenoid accumulation during the regreening in plants.

Author contributions

Masaya Kato, Masaki Yahata, Gang Ma, Lancui Zhang and Nichapat Keawmanee: conceived and designed the experiments. **Nichapat Keawmanee**: wrote the paper. **Nichapat Keawmanee**: carried out the experiments and analyzed the data. **Kan Murakami, Masashi Yamamoto, Nami Kojima**: contributed to sample collection and carried out the experiments. All authors approved the final revision to be published.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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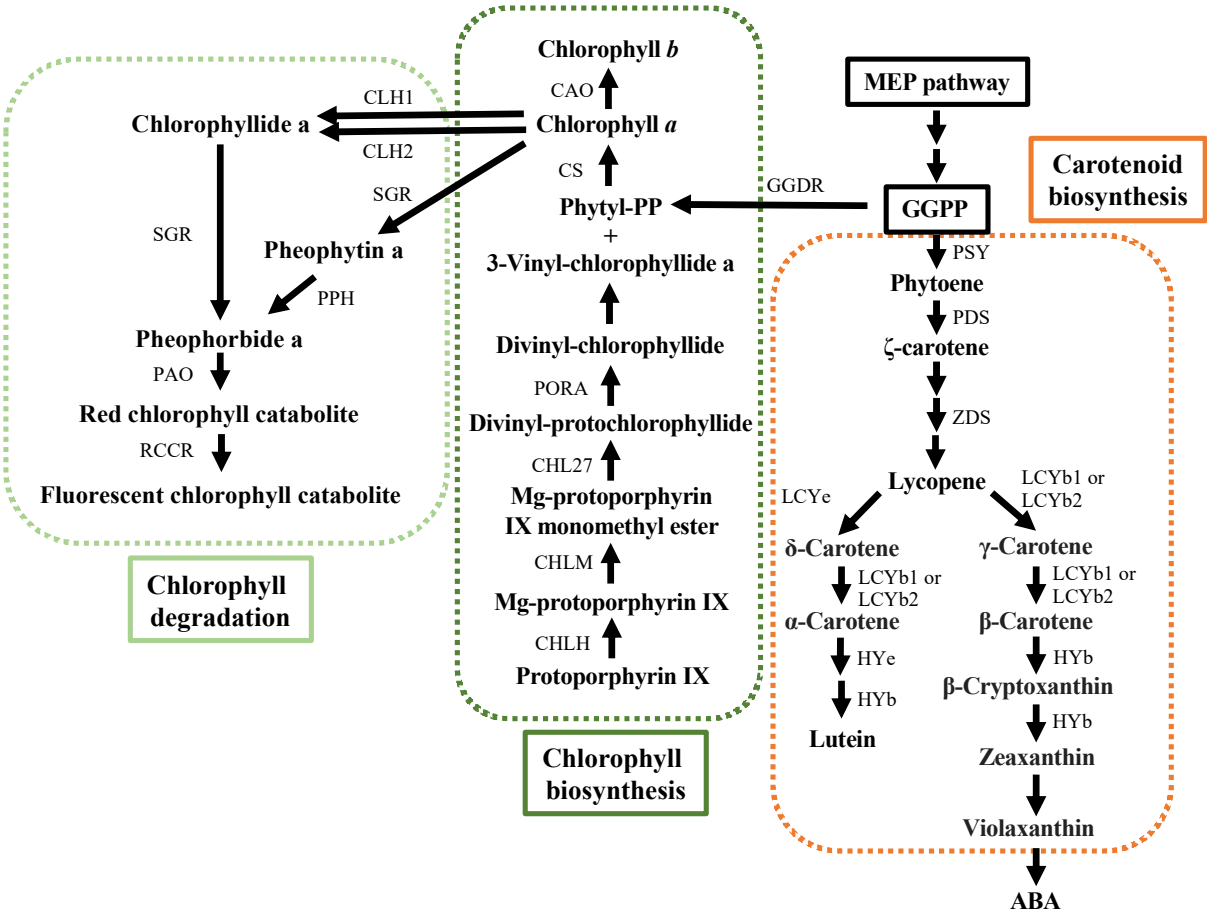


Figure 1. Metabolic pathways involved in biosyntheses of carotenoid and chlorophyll via the MEP pathway in plants. MEP pathway, methylerthritol-4-phosphate pathway; GGPP, geranylgeranyl diphosphate. The enzymes investigated in this study are: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; HYb, β-ring hydroxylase; HYe, ε-ring hydroxylase; GGDR, geranylgeranyl reductase; CHLH, magnesium chelatase; CHLM, magnesium-protoporphyrin IX methyltransferase; CHL27, Mg-Proto IX monomethyl ester cyclase; PORA, protochlorophyllide oxidoreductase a; CS, chlorophyll synthase; CAO, chlide a oxygeanase; CLH, chlorophyllase; SGR, Stay-Green; PPH, pheophytin pheophorbide hydrolase; PAO, pheophorbide a oxygenase; RCCR, Red chlorophyll catabolite reductase.

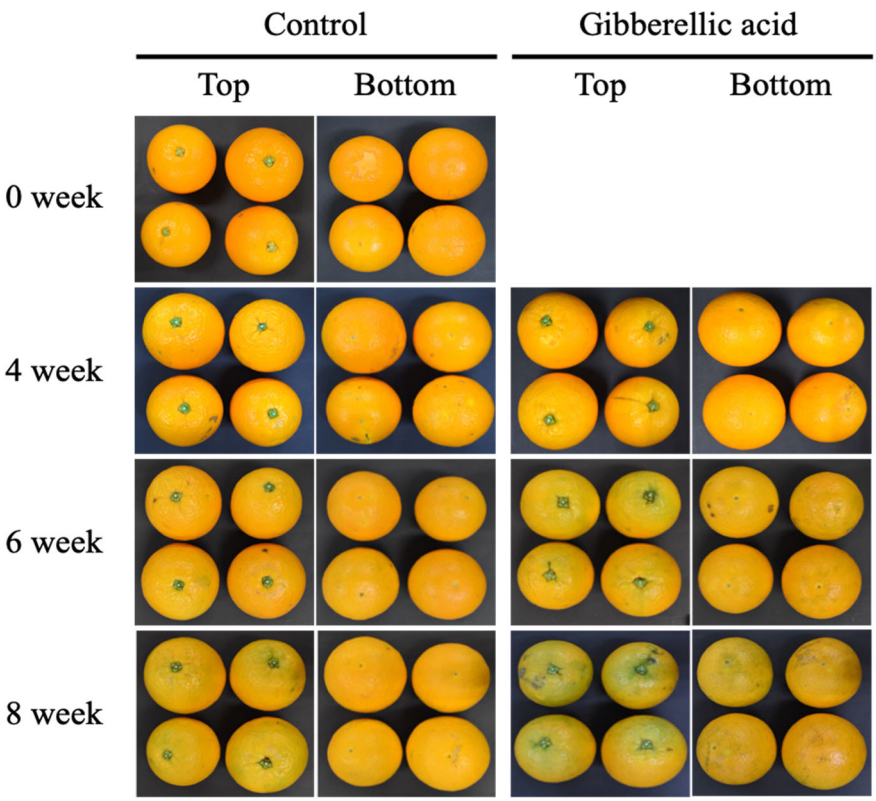
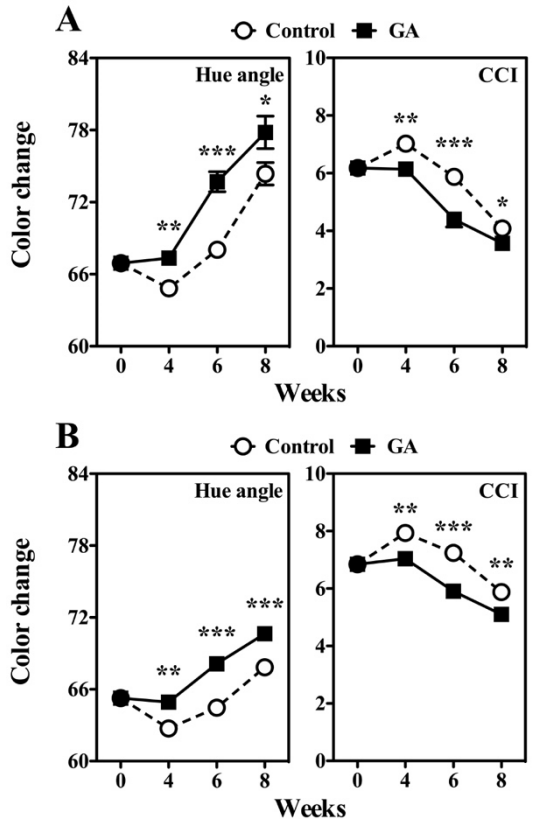


Figure 2. The appearance of GA-treated and non-treated Valencia orange fruits during the regreening process.



533

534 **Figure 3.** The values of Hue angle and citrus color index (CCI) in the top (A) and bottom (B) parts of
535 Valencia orange flavedos treated with or without GA. The results shown are the mean ± SE (n=8). The *t*-test
536 was applied to analyze the difference between the GA-treated and non-treated fruits at $*p < 0.05$; $**p < 0.01$;
537 and $***p < 0.001$ levels.

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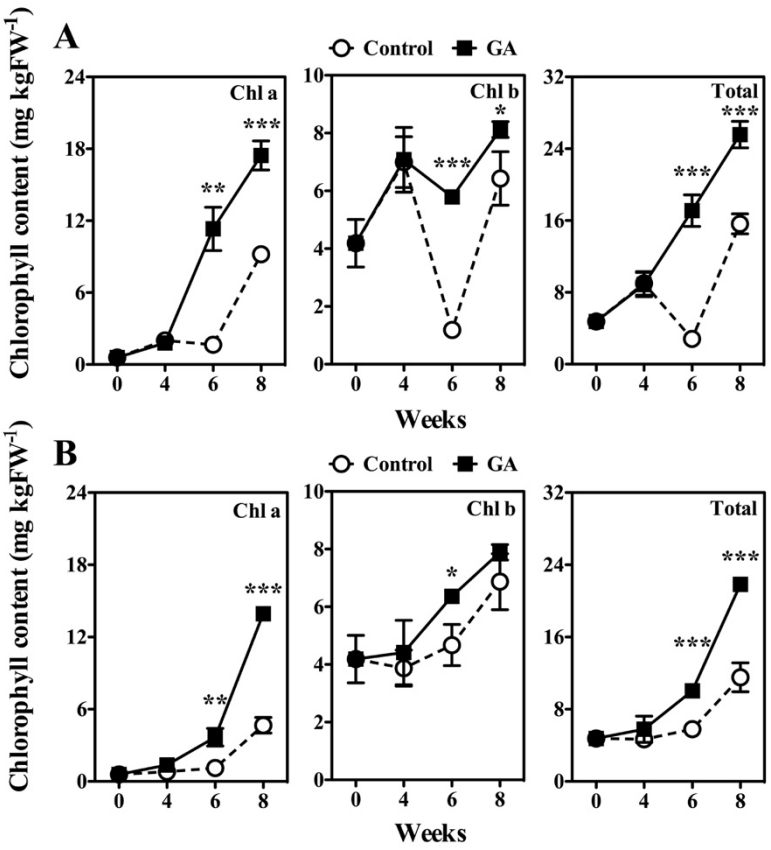

548

549 **Figure 4.** The values of chlorophyll content (Chl a, chlorophyll *a*; Chl b, chlorophyll *b*; Total, total
550 chlorophylls) in the top (A) and bottom (B) parts of Valencia orange flavedos treated with or without GA.
551 The results shown are the mean ± SE for three replicates. The *t*-test was applied to analyze the difference
552 between the GA-treated and non-treated fruits at $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ levels.

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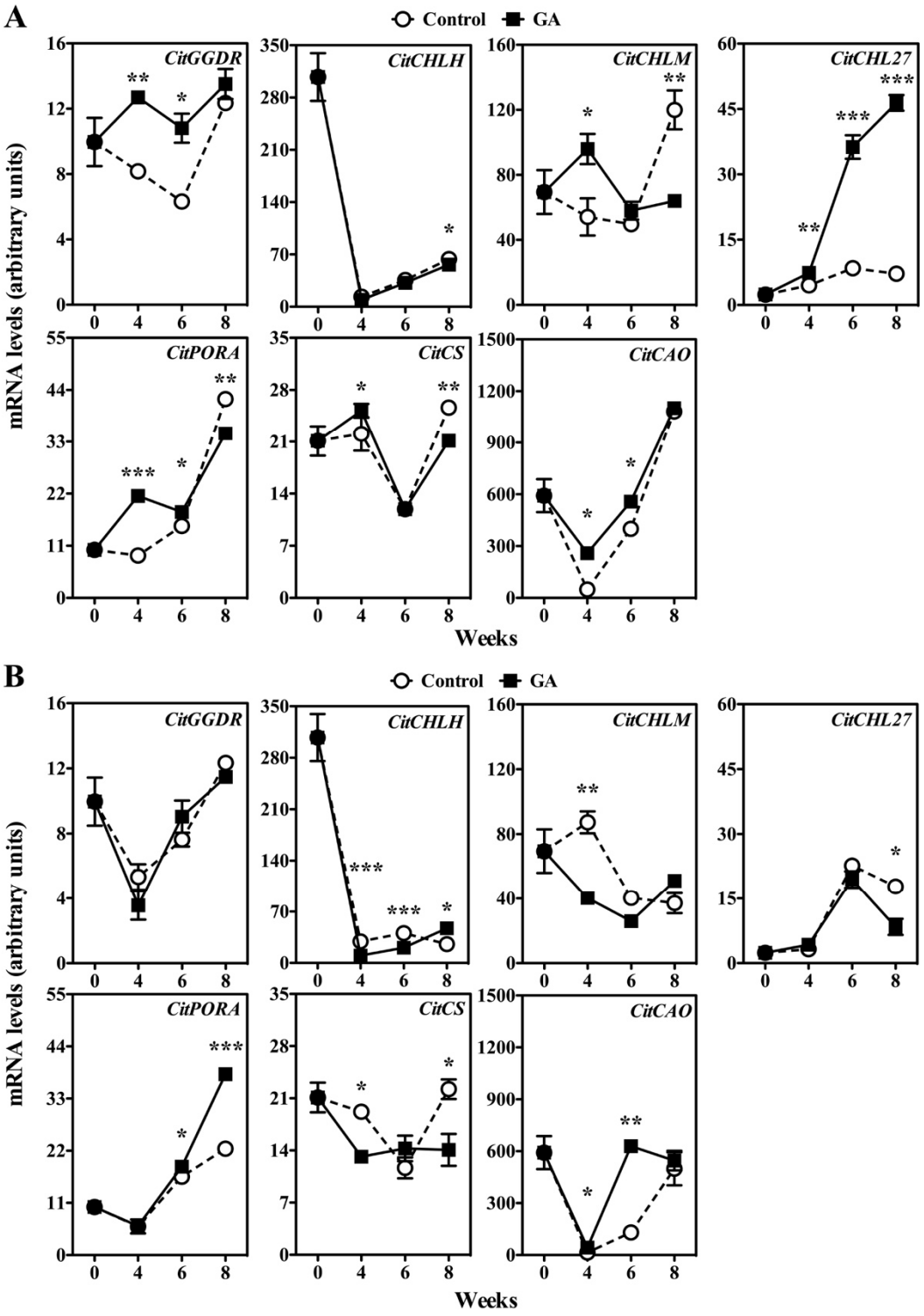
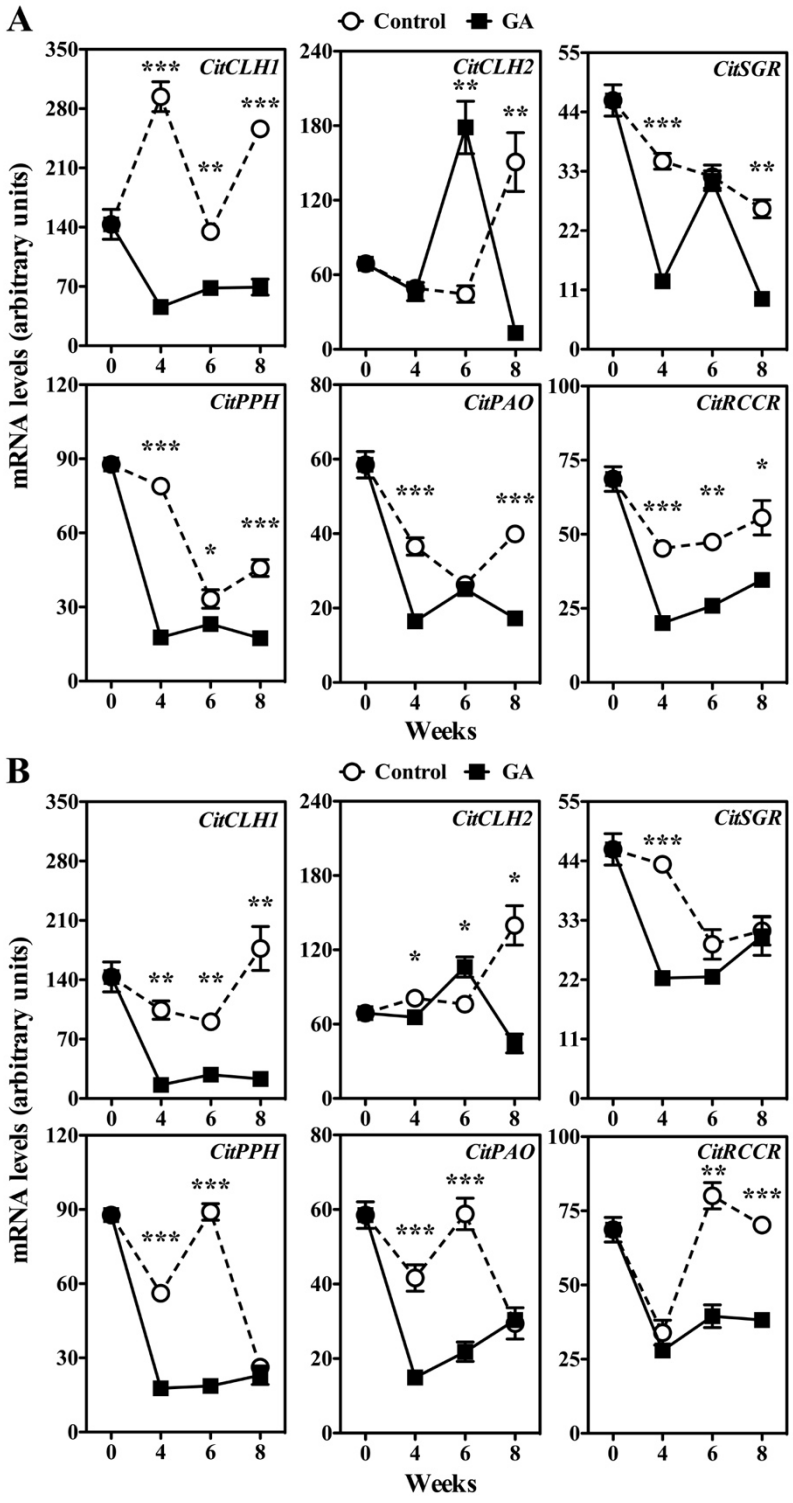


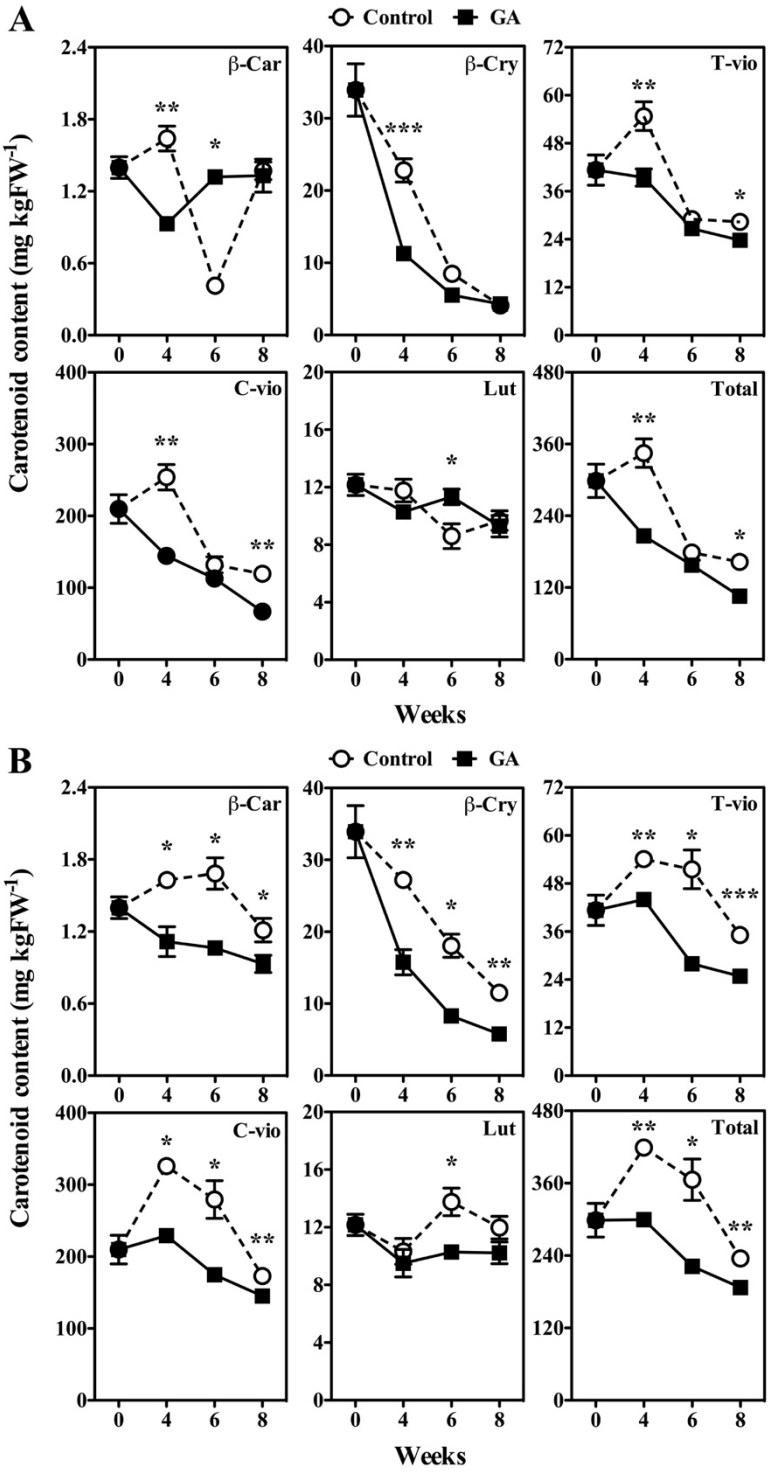
554

555 **Figure 5.** The expression of chlorophyll biosynthesis genes in the top (A) and bottom (B) parts of Valencia
556 orange flavedos treated with or without GA. The results shown are the mean ± SE for three replicates. The
557 *t*-test was applied to analyze the difference between the GA-treated and non-treated fruits at *$p < 0.05$; **p
558 < 0.01; and ***$p < 0.001$ levels.



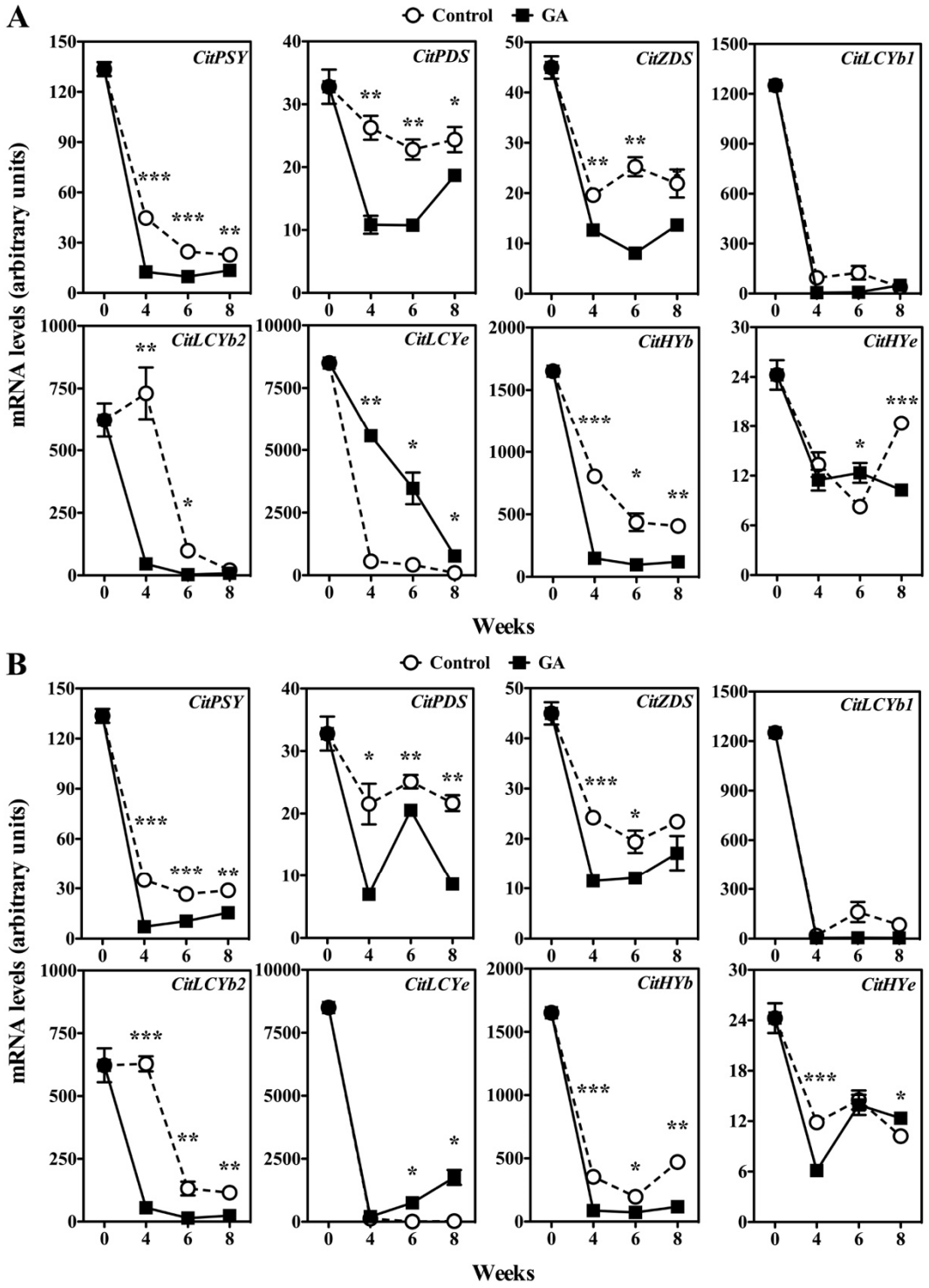
559

560 **Figure 6.** The expression of chlorophyll degradation genes in the top (A) and bottom (B) parts of Valencia
561 orange flavedos treated with or without GA. The results shown are the mean ± SE for three replicates. The
562 *t*-test was applied to analyze the difference between the GA-treated and non-treated fruits at *$p < 0.05$; **p
563 < 0.01; and ***$p < 0.001$ levels.



564

565 **Figure 7.** The values of carotenoid content in the top (A) and bottom (B) parts of Valencia orange flavedos
566 treated with or without GA. β-Car, β-carotene; β-Cry, β-cryptoxanthin; T-vio, all-*trans*-violaxanthin; C-vio,
567 9-*cis*-violaxanthin; Lut, lutein; Total, total carotenoid. The results shown are the mean ± SE for three
568 replicates. The *t*-test was applied to analyze the difference between the GA-treated and non-treated fruits at
569 $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ levels.

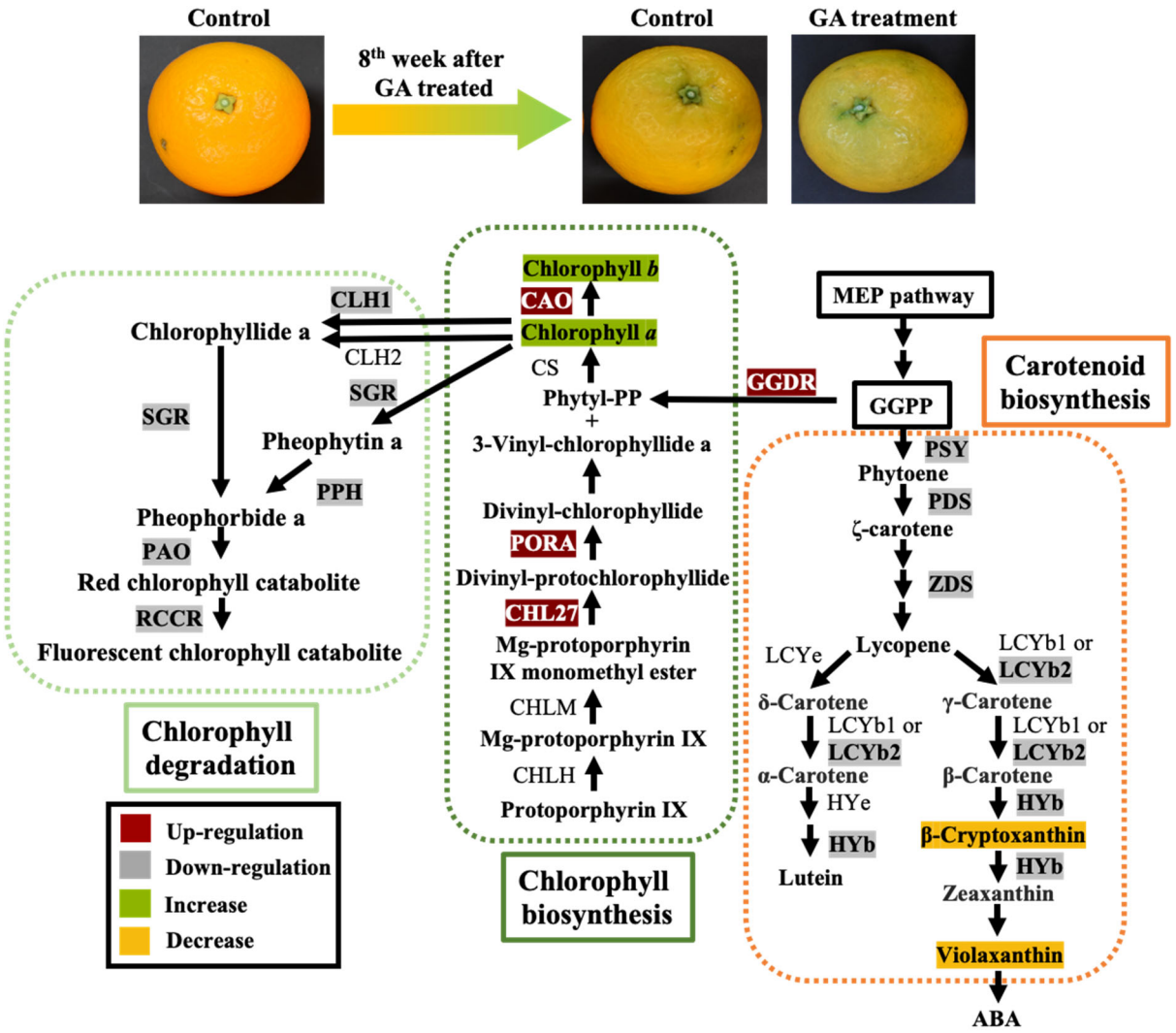


570

571 **Figure 8.** The expression of carotenoid biosynthesis genes in the top (A) and bottom (B) parts of Valencia
572 orange flavedos treated with or without GA. The results shown are the mean ± SE for three replicates. The
573 *t*-test was applied to analyze the difference between the GA-treated and non-treated fruits at *$p < 0.05$; **p
574 < 0.01; and ***$p < 0.001$ levels.

575

576 **Graphical Abstract**



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