Transcriptomic and metabolic analysis uncovers the role of light quality in carotenoid accumulation of grapefruit during ripening

Liulian Huang
Southwest University
Linping Hu
Southwest University
Wenbin Kong
Chongqing Agricultural Technology Extension Station
Can Yang
Southwest University
Wanpeng Xi (xwp1999@zju.edu.cn)
southwest university

Article

Keywords: grapefruit, light, bagging, carotenoid, RNA-sequencing, WGCNA

Posted Date: August 31st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-825511/v1

Version of Record: A version of this preprint was published at Communications Biology on April 4th, 2022. See the published version at https://doi.org/10.1038/s42003-022-03270-7.

Transcriptomic and metabolic analysis uncovers the role of light quality in carotenoid accumulation of grapefruit during ripening

Running Head: Light changes carotenoid accumulation in grapefruit

Xiulian Huang[a#], Linping Hu[a#], Wenbin Kong[b], Can Yang[a], Wanpeng Xi [a*]

[a] College of Horticulture and Landscape Architecture, Southwest University, Chongqing 400716, China

[b] Chongqing Agricultural Technology Extension Station, Chongqing 401121, China

[c] Key Lab of Aromatic Plant Resources Exploitation and Utilization in Sichuan Higher Education, Yibin University, Yibin, Sichuan 644000, China

*Correspondence author

E-mail address: xwp1999@zju.edu.cn

Postal address: College of Horticulture and Landscape Architecture, Southwest University, Chongqing China 400716

[#]These authors contributed equally to this work.

Abstract

Light, a crucial environmental signal, is involved in the regulation of secondary metabolites. To understand the mechanism by which light influences carotenoid metabolism, grapefruits were bagged with four types of light-transmitting bags that altered the transmission of solar light. We showed that light-transmitting bagging induced changes in carotenoid metabolism during fruit ripening. Compared with natural light, red light (RL)-transmittance treatments significantly increased the total carotenoid content by 142%. Based on weighted gene co-expression network analysis (WGCNA), 'red', 'darkred', 'yellow', 'brown' and 'midnightblue' modules were remarkably associated with carotenoid metabolism under different light treatment. Transcriptome analysis identified the transcription factors (TFs) bHLH74/91/122, NAC56/78/90/100, MYB/MYB308, WRKY7/55, MADS29/AGL61, ERF043/118 as being involved in the regulation of carotenoid metabolism in response to RL. Under RL treatment, these TFs regulated the accumulation of carotenoids by directly modulating the expression of carotenogenic genes, including *PSY*, *Z-ISO2*, *ZDS6*, *LCYB*, *LCYE*, *CHYB*, *CCD1-1/1-3*, *CCD4-2* and *NCED2/3*. Based on these results, a network of the regulation of carotenoid metabolism by light in citrus fruits was preliminarily proposed. These results showed that RL treatments have great potential to improve coloration and nutritional quality of citrus fruits.

Keywords: grapefruit, light, bagging, carotenoid, RNA-sequencing, WGCNA.

Introduction

Carotenoids are a large class of natural lipid-soluble pigments that are extensively distributed in plants and play important roles in plant growth and development, being involved in photosynthesis, photomorphogenesis, photoprotection, and phytohormone synthesis[1,2]. The accumulation of carotenoids confers on many fruits and vegetables their various colors, such as yellow, orange, and red hues[3]. In addition, their catabolites provide precursors for the synthesis of abscisic acid (ABA) and strigolactones (SLs), which participate in various biological processes and stress responses[4]. In humans, carotenoids in plant-based foods are an important source of dietary vitamin A, which is essential for health and nutrition, and carotenoid-rich diets are correlated with a significant reduction in the risks of chronic diseases such as cancers, cardiovascular diseases, and several degenerative diseases[2,5]. The concentrations of carotenoids in fleshy fruits thus greatly influence their commercial and nutritional value.

The grapefruit (*Citrus paradisi* Macf.) is an economically important tropical cultivated citrus fruit[6]. In 2018, the planted area of grapefruit in China was about 9200 hectares, about 25% of the global planted area, while annual production (around 5 million tons) accounted for approximately 54% of global output, indicating that grapefruit is an important part of China's citrus production (FAO statistics, http://www.fao.org/home/en/). Red grapefruit is becoming more and more preferred by consumers for its unique flavor and attractive pulp color. Besides having an abundance of a wide variety of health-promoting compounds such as flavonoids, dietary fiber, and vitamin C[7], grapefruits are richer in carotenoids than other citrus species and thus

represent an ideal material for investigating carotenoid metabolism.

The pathway of carotenoid biosynthesis has been clearly established in plants[8]. The five-carbon prenyl diphosphate isopentenyl diphosphate (*IPP*) and its double-bond isomer dimethylallyl diphosphate (*DMAPP*) are synthesized in plastids via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The subsequent condensation of two molecules of geranylgeranyl diphosphate (*GGPP*), produced from *IPP* and *DMAPP*, by phytoene synthase (*PSY*) generates the colorless 15-*cis*-phytoene. After sequential desaturation and isomerization reactions catalyzed by phytoene desaturase (*PDS*) and ζ-carotene desaturase (*ZDS*), and ζ-carotene isomerase (*Z-ISO*) and ζ-carotenoid isomerase (*CRTISO*), respectively, phytoene is converted into the red all-trans-lycopene[9]. The production of α- and β-carotene from lycopene involves a set of cyclization reactions catalyzed by lycopene ε-cyclase (*LCYE*) and lycopene β-cyclase (*LCYB*) or *LCYB* alone, representing the β, ε- and β, β-branches of the pathway, respectively, and this step is the pivotal branch point in carotenoid metabolism. Next, α-carotene is converted into lutein by β-ring hydroxylase (*CYP97A*) and ε-ring hydroxylase (*CYP97C*) of the cytochrome P450 family. The production of zeaxanthin from β-carotene is catalyzed by β-carotene hydroxylase (*CHYB*), and violaxanthin is generated via antheraxanthin by zeaxanthin epoxidase (*ZEP*). The cleavage of carotenoids is catalyzed by the proteins of carotenoid-cleavage genes (*CCD* or *NCED*), producing apocarotenoids such as β-ionone, β-citraurin, and ABA[1,3].

Carotenoid biosynthesis and degradation are coordinated by a range of enzymes encoded by structure genes and transcription factors (TFs)[8]. These structure genes have

been identified and isolated in many plant species to date[3,8]. However, only a few transcription factors related to carotenoid metabolism have been identified in plants, including RIPENING INHIBITOR (RIN) and FRUITFULL1/2 (FUL1/2) in the MADS-box family; PIF1, TOMATO AGAMOUS LIKE1 (TAGL1), SlMADS1, SlNAC1/4, SlAP2a, SlERF6, and SlBBX20 in tomatoes[10-12]; CsMADS5/6 and CrMYB68 in oranges (flavedo)[13-15]; CpEIN3a, CpNAC1/2, and CpbHLH1/2 in papaya[16-18]; AdMYB7 in kiwifruits[19]; and R2R3-MYB subgroup Reduced Carotenoid Pigmentation 1/2 (RCP1/2) in monkey flower species[12,20]. Compared with that of anthocyanin metabolism, the transcriptional regulation of carotenoid metabolism is far from understood.

Light not only provides the energy required for photosynthesis but also participates in the regulation of a variety of metabolic processes as the crucial medium for the exchange of information between the plant and external environment during development[21,22]. An increasing number of investigations suggest that light signals also play a fundamental role in secondary metabolism in fruit. However, the majority of studies have focused on the effect of postharvest light treatment on fruit quality, with only a few referring to the impact of developmental light treatment on fruit quality[23]. As an effective method of protecting fruit from insect infestations, bird attack, and sunburn as well as reducing disease incidence rate and chemical residues, fruit bagging is extensively used in modern orchards[24]. Light-transmitting paper bags of different colors can absorb the light waves of the corresponding colors, making their use a feasible approach for investigation of how light influences phytochemicals metabolism

during fruit development.

This study was carried out to know the role of light quality on the carotenoid accumulation in grapefruit and understand the transcriptional regulatory mechanism underlying light signals during fruit ripening. The carotenoid level of 'Huoyan' grapefruit pulp treated with different light-transmittance during the ripening were compared, WGCNA were employed to identify the key genes and TFs responsible for carotenoid metabolism during the process. Based these results, a regulatory network of carotenoid metabolism in response to red-light was preliminarily proposed. These findings provide new insight into carotenoid metabolism and demonstrate a potential approach for improvement of the coloration and nutritional quality of citrus fruit and other horticultural crops.

Results

Effects of light transmittance on TSS, TA, and CCI during fruit ripening

Compared with that in the dark shade treatment (DS), the TSS content of the grapefruits treated with RL, BL, and WL gradually increased during fruit ripening and were significantly higher than that in DS treatment at 215 DAB ($p < 0.05$) (**Fig. 1A**). The effect of light treatment on TA content is different (**Fig. 1B**). It is worth noting that the TA content under RL and WL treatment slightly rose during fruit ripening while the TA content gradually decreased in BL and DS treatment. CCI shared similar trends and were gradually increased in all light treatments (**Fig. 1C**).

Effects of light transmittance on carotenoid accumulation during fruit ripening

Five major carotenoids were identified from 'Huoyan' grapefruit pulp, including β-carotene, phytofluene, ζ-carotene, lycopene, and 9-*cis*-violaxanthin (**Table S1**). The carotenoid profiles differed in the four light treatments, and the 'Huoyan' grapefruit pulp was rich in β-carotene and lycopene (**Fig. 2**). Compared with the control group (DS), the total carotenoid content was the highest in the grapefruits treated with RL (2.4-fold), followed by the grapefruits treated with BL (1.69-fold) and WL (1.59-fold) at 215 DAB. Subsequently, we found that the content of β-carotene, ζ-carotene lycopene and phytofluene gradually increased as fruit ripening under different light treatments while 9-*cis*-violaxanthin content didn't show marked changes. Compared with DS, the β-carotene and lycopene content significantly increased under RL treatment and remarkably higher than those in BL and WL treatment at 215 DAB.

Transcriptome profiles during fruit ripening

Average clean reads number of mRNA libraries for eight samples ranged from 20.98 to 23.09 million (**Table S2**). The alignment of the clean reads against the reference genome and reference gene sequences generated a total of 25,694 unigenes (**Table S3**). In four light treatments, the median of gene expression level ranged from 0.76 to 0.88 and there were differences between the samples (**Fig. S2**). The fruit samples in BL treatment showed the lowest median, 0.76, at 215 DAB, while the samples treated with WL presented the biggest median value of 0.88 at 185 DAB. However, in the DS and RL treatment groups, the gene expression levels for simples were relatively stable

during ripening.

Identification of differentially expressed genes (DEGs)

Based on RNA sequencing results, a total of 5541 DEGs were identified during fruit ripening, with 2305, 1381 and 1855 DEGs showing differential expression between DS and RL, DS and BL, and DS and WL, respectively (**Fig. 3A**). In the RL group, 1817 and 488 DEGs were identified at 185 and 215 DAB, respectively, and the number of DEGs was significantly higher than other light treatments. The numbers of extremely significant DEGs throughout fruit ripening were 152, 167 and 173, respectively, after RL, BL, and WL treatment (**Fig. 3B**). Notably, at the two indicated detection points, the unique DEGs of RL, BL and WL treatment, respectively, reached a maximum of 1650 at 185 DAB, 598 DEGs at 215 DAB and 1159 DEGs at 185 DAB.

Weighted gene co-expression network analysis (WGCNA)

The WGCNA was performed using 12619 unigenes (FPKM > 1, the top 50% of variance), which were classified into twenty-four modules (**Fig. 4**), of which the 'red', 'darkred', 'yellow', 'brown' and 'midnightblue modules were remarkably associated with carotenoid metabolism under different light condition during fruit ripening ($p < 0.05$). The analysis of the correlation between gene expression and carotenoid accumulation demonstrated that the 'red' and 'darkred' module, respectively, contained 469 (3.7%) and 53 (0.4%) genes and was significantly positively correlated with the content of carotenoid. Besides, 'yellow' module was positively associated with

phytofluence content (r=0.78, *p*=0.02). However, 'brown' and 'midnightblue' modules were highly negatively related with phytofluence content, which contained 1882 genes (14.9%), with a correlation coefficient of −0.75 (p = 0.03) and −0.72 (p = 0.04), respectively. These results indicate that genes in these modules were potentially correlated with carotenoid accumulation under different light conditions.

Expression analysis of genes related to carotenoid metabolism

In the carotenoid metabolic pathway (**Fig. 5a**), a total of eight structural genes, including *GGPPS1*, *ZEP1*/*2*, *CCD1-1*/*1-3*, *CCD4-1*, *NCED2*/*3*, were differentially expressed in response to RL during ripening (**Table S4**). RL significantly induced the transcription for carotenoid biosynthetic and cleavage genes during grapefruit ripening (**Fig. 5A**). Among the 3080 genes in the carotenoid co-expression modules, a total of 235 transcription factors (TFs), which were enriched in 52 gene families such as ERF (21), bHLH (19), MYB (18), WRKY (15), NAC (9) and MADS (7), were identified (**Fig. S3**). In order to further excavate the transcription factors responding to RL, we screened the differentially expressed transcription factors between RL and DS group. In order to further excavate the transcription factors responding to RL, we screened the differentially expressed transcription factors between RL and DS group. Subsequently, a total of forty-eight differentially expressed TFs in response to RL, including ERF (13), WRKY (7), bHLH (5), NAC (4), C3H (4), bZIP (4), MYB (3), GRAS (3), HSF (2), Dof (2), MADS (2) and GRF (1), were identified as candidate TFs modulating carotenoid biosynthesis in response to RL during fruit ripening (**Fig. 5B, Table S5**).

Visualization of gene networks

In order to identify the hub gene underlying carotenoid metabolism under different light-transmittance conditions, the co-expression for structural genes and regulators was visualized using Cytoscape. In carotenoid co-expression modules, forty TF members—derived from the ERF (10), WRKY (9), bHLH (5), bZIP (4), NAC (3), MYB (3), C3H (2) Dof (2), GRAS (1) and GRF (1) families—were identified as the key genes related to carotenoid metabolism. Meanwhile, twelve structural genes, namely *IPI*, *PDS*, *ZDS4*/*ZDS7*, *CRTISO1*/*3*, *CYP97C1*/*2*, *CCD1-2*, *CCD4-2*, *CCS*, *NXS*, which directly involved in the carotenoid biosynthesis and cleavage, were identified as the key regulatory genes in co-expression network (**Fig. 6**). In addition, we found that thirty-two TFs, including ERF, WRKY, bHLH, NAC and MYB family members were co-expressed with carotenoid cleavage gene *CCD4-2* and six TFs had a co-expression relationship with *CYP97C1* (**Table S6**). Notably, there was a co-expression relationship between MYB4 and *CCD1-2*, *CCS*, *PDS*. These results suggested that above transcription factors and structural genes might interact with each other to regulate the flux for carotenoid in grapefruits.

Discussion

Light signals play a vital role in carotenoid metabolism.[22,25] Although many studies have investigated the effects of postharvest light treatments, such as LED, pulse, and ultraviolet light, on carotenoid metabolism[26-28], the role of light quality in carotenoid

226 metabolism underlying fruit ripening previously had not been elucidated. In this work,
227 different light-transmittance bagging treatments were used to understand the influence
228 of light quality on grapefruits throughout the ripening process at the metabolic and
229 molecular levels.

230 Existing research indicates light irradiation modulates the biosynthesis and
231 catabolism of carotenoids in fruit and modifies the concentration and composition of
232 carotenoids[25,29]. The total carotenoid content was reduced upon ripening in covered
233 tomatoes and peppers[23]. In grapefruit peel, an unusual pattern of lycopene accumulation
234 in might be associated with the developmentally regulated differentiation of
235 chromoplasts mediated by light[29]. Light deprivation promoted peel degreening and
236 reduced carotenoid accumulation in mandarins and sweet orange fruits[25]. In contrast,
237 light irradiation enhanced carotenoid accumulation and external quality during
238 mandarin fruit development[30]. Here, our results showed that carotenoid (total
239 carotenoid, β-carotene, phytofluene, and lycopene) accumulation was significantly
240 induced by red-transmittance bagging treatments (**Fig. 2**), suggesting that RL played
241 significant positive role in carotenoid accumulation in grapefruit pulp. These are
242 evidence of the promotion of carotenoid metabolism by light in fruit.

243 Lighting factors can regulate secondary metabolism by light quality, light intensity,
244 and light irradiation time in plant[23]. Red LED light (660 nm) activated the expression
245 of *VvNCED1* in ripening grape skin (*Vitis vinifera* L.)[31], but had no effect on the
246 carotenoid content of citrus juice sacs. Blue LED light (470 nm) treatment stimulated
247 carotenoid accumulation by upregulating the expression of the *CitPSY* gene in the juice

248 sacs of three citrus varieties (mandarin (*Citrus unshiu* Marc.), Valencia orange (*C.*
249 *sinensis* Osbeck) and Lisbon lemon (*C. limon* Burm.f.) [32]. In the present study, we found
250 that RL promoted lycopene and β-carotene accumulation along with upregulation of
251 *PSY*, *Z-ISO1/2*, *ZDS1/6*, *LCYB*, *LCYE*, *NCED3* and *CCD1-3* (**Fig. 2 and 5**), which was
252 similar with the upregulation of the *PSY* gene induced by continuous red light in
253 *Arabidopsis thaliana* seedlings, leading to increases in carotenoid content[33]. However,
254 blue- and white-light transmittance treatment have no significant effect on carotenoid
255 content during grapefruit ripening. These results revealed that the regulatory roles of
256 light in carotenoid metabolism also depends on the light quality.

257 A large number of TFs have been reported to be involved in carotenoid metabolism
258 via transcriptional regulation of key structural genes in plant. Here, multiple members
259 of ERF, NAC, WRKY, MYB, MADS, bHLH families be identified as hub genes for
260 modulating carotenoid flux (**Fig. 5 and 6**). Work in *Arabidopsis thaliana* revealed that
261 the PIF1 (phytochrome interacting factor 1) transcription factor suppressed *PSY*
262 transcript by directly binding the G-box motifs and further regulated carotenoid
263 metabolism[34]. Zhou et al. showed that CpbHLH1/2 promoted lycopene degradation to
264 carotenoids by upregulating the transcripts of the lycopene β-cyclase genes (*CpLCYB*
265 and *CpCHYB*) in response to strong light during papaya ripening[18]. Blue- and red-light
266 supplementation irradiation to tomato fruits at anthesis facilitated lycopene biosynthesis,
267 which was considered to be related with regulation of the photoreceptor HY5
268 (ELONGATED HYPOCOTYL5) and PIFs upon the expression of *PSY*1[35]. Here, in RL-
269 treated grapefruit, bHLH62/74/91/122 in 'brown' module negatively correlated with

phytofluene presented markedly down-regulated trend coupled with transcript increase of *PSY* and CHYB (**Fig. 2, 5 and 7**), which accounted for higher phytofluene level, and these results are paralleled with carotenoid increase of content in SlPIF4-silenced tomato[36].

Some ripening related regulators have been shown the regulatory role in carotenoid metabolism. In tomato, MADS-box TFs RIN gene was reported to specifically regulating accumulation of lycopene by positively regulated carotenoid biosynthetic genes (including *PSY*, *Z-ISO*, *CRTISO*) and negatively regulated carotenoid downstream genes *LCYB* and *LCYE*, while FUL homologs FUL1/2 regulated overall carotenoid pathway by targeted multiple carotenogenic genes[37,38]. In sweet orange (*Citrus sinensis*), CsMADS5/6 activated expression for carotenogenic genes, including *PSY*, *PDS*, *LCYb1*/*CCD1* via directly binding its promoter and thus modulated carotenoid metabolism[13,14]. In 'darkred' module, we found that up-regulation of MADS29 in response to RL was accompanied by the increased expression level of *Z-ISO2*, *LCYB* as well as *CCD1-3*, which facilitated carotenoid accumulation (**Fig. 2, 5 and 7**). By contrast, the significantly reduced expression of AGL61 for 'darkred' module during ripening suggested their negative correlation with phytofluene accumulation in grapefruit (**Fig. 5 and 7**). Recently, PpERF3 has been shown to be involved in ABA biosynthesis by activating *PpNCED2/3* transcription during peach fruit ripening[39]. Here, PpERF3 homolog ERF012 was down-regulated in response to RL, which suggested ERF012 were highly likely to be involved in carotenoid process mediated by RL. On the contrary, RL remarkably promoted transcript of ERF043/118

292 and *NCED2/3* shared similar expression patterns with them (**Fig.5 and 7**). Above
293 analysis indicated ERF TFs differently respond to RL and collaboratively regulated
294 carotenoid accumulation. In the *Arabidopsis*, suppression of AtRAP2.2 leads to
295 reduction of *PSY* and *PDS* transcript[40]. In rice leaves, AP2/ERF genes were negatively
296 associated with carotenoid accumulation under both blue- and red-light treatments[41].
297 Here, we also found multiple ERF TFs (ERF023-like/025/026) in 'brown' module
298 displayed negative correlation with phytofluence accumulation in response to RL.

299 Another fruit ripening related TF NACs were also reported to be involved in
300 carotenoid metabolism. In tomato SlNAC4/19/48 RNAi fruit, the transcript levels of
301 *PSY* were reduced and thus resulted in decreased lycopene[42,43]. However, the
302 overexpression of SlNAC1 reduced lycopene content, which was associated with a
303 reduction in *SlPSY* and an increase in *SlLCYB* and *SlLCYE* expression[44]. During papaya
304 fruit ripening, CpNAC2 co-operated with CpEIN3a to promote *CpPDS2/4*, *CpZDS*,
305 *CpCHYB*, and *CpLCYE* transcription, accounting for the elevated carotenoid contents[17].
306 CcNAC1/2 were transcriptionally upregulated under red-light treatment in *Citrullus*
307 *colocynthis*[45]. Similarly, FcrNAC22 upregulated carotenoid metabolism and ABA
308 synthesis via activation of *FcrLCYB1*, *FcrBCH2* and *FcrNCED5* in RL-irradiated
309 fruits[46]. Here, we observed that the increased expression levels of NAC56 (the
310 SlNAC48 homolog) and NAC100 mediated by RL were positively related with
311 expression of up-stream genes (*PSY*, *Z-ISO2*, *ZDS6*, *LCYB* and *LCYE*) in carotenoid
312 metabolic pathway, while downregulated NAC68/78/90 showed a negative correlation

313 with transcript for these genes, consistent with lycopene accumulation in ripening
314 grapefruit fruit (**Fig. 2, 5 and 7; Fig. S4**).

315 Some publications in recent years have reported that MYB TFs played a positive
316 role in carotenoid regulation. In the flavedo of *Citrus reticulate*, CrMYB68 indirectly
317 inhibited the transformation of α/β-carotene via negative regulation for *CrBCH2* and
318 *CrNCED5*[15]. AdMYB7 was positively correlated with *AdLCYB* in terms of expression
319 and further regulated carotenoid biosynthesis[19]. In 'darkred' module, we found that
320 MYB308 induced by RL was also positively correlation with carotenoid accumulation,
321 especially lycopene, during grapefruit ripening (**Fig. 4 and 5**). Additionally, seven
322 WRKY TFs were differentially expressed in response to RL during grapefruit ripening
323 (**Fig. 5**). In *Osmanthus fragrans*, OfWRKY3 was found to be a positive regulator of the
324 *OfCCD4* gene via binding to its W-box palindrome motif[47]. In this study, we also
325 observed that two WRKY TFs, namely WRKY7/55 were gradually down-regulated as
326 grapefruit fruit ripening, accompanied by the reduction of *CCD4-2* expression of in RL
327 treatment (**Fig. 2, 5 and 7**). Besides, RL also notably suppressed expression for C3H
328 (2), bZIP (4) and Dof (2) TFs in 'brown' module, suggested these TFs might involve in
329 carotenoid accumulation (**Fig. 5**).

330

331 **Conclusion**

332 In present study, the carotenoid accumulation in grapefruit respond differently to light
333 quality, RL have the significant inducing role during fruit ripening. The process was
334 modulated by multiple TFs (bHLH74/91/122, NAC56/78/90/100, MYB/MYB308,

335 WRKY7/55, MADS29/AGL61, ERF043/118,) as well as carotenogenic genes (*PSY*, *Z-*
336 *ISO2*, *ZDS6*, *LCYB*, *LCYE*, *CHYB*, *CCD1-1/1-3*, *CCD4-2* and *NCED2/3*), and a
337 preliminary regulatory model of red light-induced carotenoid metabolism in grapefruits
338 was established (**Fig. 7**). These findings not only provide new insight into the regulation
339 of carotenoid metabolism, but also offer an effective approach for enhancing the quality
340 of citrus fruits in agricultural practice.
341
342 **Materials and methods**
343 **Plant materials and treatments**
344 'Huoyan' grapefruit were cultivated at the National Citrus Germplasm Repository of
345 the Citrus Research Institute at the Chinese Academy of Agricultural Sciences in
346 Chongqing, China and used as experimental materials. Trees with the same age, tree
347 structure, and identical growth conditions were selected for the experiment and
348 cultivated under the same management condition. Grapefruits with similar sizes and
349 colors from outside of tree were bagged with four different light-transmitting paper bags
350 at 120 days after blossom (DAB)—red-light-transmitting bags (RL) (peak wavelength,
351 748 nm), blue-light-transmitting bags (BL) (peak wavelength, 478 nm), and white-
352 light-transmitting bags (WL), and a dark-shading bag (DS) was as the control (**Fig. S1**).
353 Fruits of a uniform size were picked at 185 (maturation) and 215 (fully ripe) days after
354 blossom (DAB). Each fifteen fruits were as one replicate and three biological replicates
355 were used for each sample point of every treatment. After determining the basic
356 physiological parameters, the fruits were cut into small cubes, frozen using liquid

nitrogen, and stored at −80 °C for further analysis.

Determination of basic physiological parameters

The fruit color parameters were measured using the Hunter Associates Laboratory Scanner (Hunter Associates Laboratory, Inc., Reston, VA, USA). The citrus color index (CCI) for the mesocarp was calculated according to the formula CCI = 1000 × a* / (L* × b*), using five fruits as a single replicate and three biological replicates were used for each sample. To determine the total soluble solid (TSS) content, 200 μL of fresh squeezed juice was obtained from juice sacs and then analyzed with a digital hand-held refractometer (Atago PR-101R, Atago, Japan). Titratable acidity (TA) was measured after the juice sample was diluted 50 times with purified water.

Extraction and identification of carotenoids

Carotenoids were identified following our previously described method[48]. Ten grams of pulp powder was extracted with 20 mL of solvent (hexane/acetone/ethanol, 50:25:25, v/v/v) in a screw-top tube. The colored top layer was recovered and dried with nitrogen gas after being left to stand for 30 min, protected from light. After saponification, 2 mL of 1% butylhydroxytoluene (BHT)/methyl tert-butyl ether (MTBE) was added to the colored layer, and the mixture was filtered through sodium sulfate into a brown bottle for drying. The residue was dissolved in 2 mL of methanol/acetone (2:1, v/v) for HPLC analysis.

The carotenoids were identified by HPLC (Waters, Milford, MA, USA) with a C_{30}

chromatography column (250 × 4.6 mm, 5 μm; YMC, Wilmington, NC, USA). The mobile phases for the carotenoids were composed of MTBE (A), methanol (B), and an aqueous phase (C) and were prepared by a multistep linear gradient elution. The identification was performed by comparing the retention times and UV–visible spectral peaks between the samples and standards. The carotenoid contents were calculated according to a standard curve based on authentic compounds and are expressed herein as mg/kg fresh weight (FW).

Library construction, transcriptome sequencing, and gene annotation

Total RNA was extracted using an Agilent RNA 6000 Nano kit (Agilent, CA, USA) according to the manufacturer's instructions, the RNA concentration and integrity were assessed using an Agilent 2100 Bioanalyzer, and the OD260/OD280 and OD260/OD230 values were determined using a NanoDrop 2000 spectrophotometer (NanoDrop 2000, Wilmington, DC, USA) to assess the RNA purity. Eight mRNA libraries were constructed for RNA-seq of the pulp samples harvested at 185 and 215 DAB. Three biological replicates were performed for each sample.

The libraries were sequenced on an Illumina HiSeq™ 2000 system at the Beijing Genomics Institute (BGI), China. The raw sequencing data were filtered by removing adaptors, low-quality and redundant sequences, and reads with unknown "N" base content higher than 5% using the SOAPnuke (version 1.4.0) and Trimmomatic (version 0.36) software. The clean reads were aligned to the reference genome database using HISAT (version 2.1.0)[49].

For transcription factor annotation, open reading frames (ORF) were obtained from the quality-checked data using getorf (EMBOSS: 6.5.7.0, http://emboss.sourceforge.net/apps/cvs/emboss/apps/getorf.html, -minsize 150) and aligned to Plant Transcription Factor Database (http://planttfdb.gao-lab.org/blast.php).

Identification of differentially expressed genes (DEGs)

The RSEM software package (version 1.2.8, http://deweylab.biostat.wisc.edu/rsem/rsem-calculate-expression.html) was used to calculated expression levels for transcripts with the default parameters[50]. The expression levels are expressed as FPKM values. The genes that were differentially expressed between two samples were determined as previously described according to the Poisson distribution and the algorithm developed by BGI[51]. The DEGs ($p \leq 0.005$; $|\log_2 \text{fold change}| \geq 1$; FDR ≤ 0.001) were then screened for further analysis.

Weighted gene co-expression network analysis (WGCNA) and network visualization for candidate genes

A total of 16831 unigenes with FPKM values > 1 were utilized to conduct weighted gene co-expression network analysis using WGCNA[52], reshape2 and stringr packages in Rstudio (v1.4.1717, https://www.rstudio.com/products/rstudio/download/). To reduce the size of the data calculation, a total of 12619 unigenes with the first 75% variance were screened from the above unigenes with unsigned TOM type to build a co-expression network. The phenotypic data regarding the carotenoids in the pulp were

associated with the constructed co-expression network to screen the modules that were significantly correlated with carotenoid metabolism ($p \leq 0.05$). Finally, DEGs were imported into the cytoscape software (version 3.7.2, https://cytoscape.org/download.html) for network visualization.

Acknowledgements

This work was supported by the National Science Foundation of Chongqing for Distinguished Young Scholars (No. cstc2019jcyjjq0029).

Data availability

The transcriptome raw reads have been deposited as a BioProject (https://submit.ncbi.nlm.nih.gov/subs/sra/) under accession number: PRJNA728380.

Author contributions

W.X. designed the project. X.H. prepared the manuscript. L.H. analyzed the data. W.K. and W.Z participated in collecting the materials. C.Y., L.H., and X.H. participated in assaying the physiological parameters.

Competing interests

The authors declare no competing interests

Additional information

Supplementary information

References

1. Sun, T. et al. Carotenoid metabolism in plants: the role of plastids. *Mol. Plant* **11**, 58-74 (2018).
2. Rowles, J. L. & Erdman, J. W. Carotenoids and their role in cancer prevention. B*iochim. Biophys. Acta Mol. Cell Biol. Lipids* **1865**, 158613 (2020).
3. Nisar, N., Li, L., Lu, S., Khin, N. C. & Pogson, B. J. Carotenoid metabolism in plants. *Mol. Plant* **8**, 68-82 (2015).
4. Jia, K. P., Baz, L. & Al-Babili, S. From carotenoids to strigolactones, *J. Exp. Bot.* **69,** 2189-2204 (2018).
5. Fanciullino, A. L., Bidel, L. P. R. & Urban, L. Carotenoid responses to environmental stimuli: integrating redox and carbon controls into a fruit model. *Plant Cell Environ.* **37**, 273-289 (2014).
6. Corazza-Nunes, M. J., Machado, M. A. & Nunes, W. M. C. Cristofani M and Targon MLPN, Assessment of genetic variability in grapefruits (*Citrus paradisi* Macf.) and pummelos (*C. maxima* (Burm.) Merr.) using RAPD and SSR markers. *Euphytica* **126**, 169-176 (2002).
7. Zou, Z., Xi, W., Hu, Y., Nie, C. & Zhou, Z. Antioxidant activity of citrus fruits. *Food Chem.* **196**, 885-896 (2016).
8. Yuan, H., Zhang, J., Nageswaran, D. & Li, L. Carotenoid metabolism and regulation in horticultural crops. *Hortic Res.* **2**, 15036 (2015).
9. Isaacson, T., Ohad, I., Beyer, P. & Hirschberg, J. Analysis in vitro of the enzyme CRTISO establishes a poly-cis-carotenoid biosynthesis pathway in plants. *Plant*

Physiol. **136**, 4246-4255 (2004).

10. Lee, J. M. et al. Combined transcriptome, genetic diversity and metabolite profiling in tomato fruit reveals that the ethylene response factor SlERF6 plays an important role in ripening and carotenoid accumulation. *Plant J.* **70**, 191-204 (2012).
11. Llorente, B. et al. Tomato fruit carotenoid biosynthesis is adjusted to actual ripening progression by a light-dependent mechanism. *Plant J.* **85**, 107-119 (2016).
12. Stanley, L. E. et al. A tetratricopeptide repeat protein regulates carotenoid biosynthesis and chromoplast development in Monkeyflower (*Mimulus*). *Plant Cell* **32**, 1536-1555 (2020).
13. Lu, S., Ye, J. & Zhu, K. A fruit ripening-associated transcription factor CsMADS5 positively regulates carotenoid biosynthesis in citrus. *J. Exp. Bot.* **72**, 3028-43 (2021).
14. Lu, S. et al. The Citrus transcription factor CsMADS6 modulates carotenoid metabolism by directly regulating carotenogenic genes. *Plant Physiol.* **176**, 2657-2676 (2018).
15. Zhu, F. et al. An R2R3-MYB transcription factor represses the transformation of alpha- and beta-branch carotenoids by negatively regulating expression of *CrBCH2* and *CrNCED5* in flavedo of citrus reticulate. *New Phytol.* **216**, 178-192 (2017).
16. Fu, C. C. et al. The Papaya Transcription Factor CpNAC1 Modulates Carotenoid Biosynthesis through Activating Phytoene Desaturase Genes CpPDS2/4 during Fruit Ripening. *J. Agri. & Food Chem.* **64**, 5454-63 (2016).
17. Fu, C. C., Han, Y. C., Kuang, J. F., Chen, J. Y. & Lu, W. J. Papaya CpEIN3a and

491 CpNAC2 co-operatively regulate carotenoid biosynthesis-related genes CpPDS2/4,
492 CpLCY-e and CpCHY-b during fruit ripening. *Plant Cell Physiol.* **58**, 2155-2165
493 (2017).
494 18. Zhou, D. et al. Papaya CpbHLH1/2 regulate carotenoid biosynthesis-related genes
495 during papaya fruit ripening. *Hortic Res.* **6**, 80 (2019).
496 19. Ampomah-Dwamena, C., Thrimawithana, A. H., Dejnoprat, S., Lewis, D., Espley,
497 R. V. & Allan, A. C. A kiwifruit (*Actinidia deliciosa*) R2R3-MYB transcription
498 factor modulates chlorophyll and carotenoid accumulation. *New Phytol.* **221**, 309-
499 325 (2019).
500 20. Sagawa, J. M. et al. An R2R3-MYB transcription factor regulates carotenoid
501 pigmentation in Mimulus lewisii flowers. *New Phytol.* **209**, 1049-1057 (2016).
502 21. Lado, J., Zacarias, L. and Rodrigo, M. J. Regulation of Carotenoid Biosynthesis
503 During Fruit Development. *Subcell Biochem.* **79**, 161-198 (2016).
504 22. Cruz, A. B. et al. Light, ethylene and auxin signaling interaction regulates
505 carotenoid biosynthesis during tomato fruit ripening. Front *Plant Sci.* **9**, 1370
506 (2018).
507 23. Yoo, H. J., Kim, J. H., Park, K. S., Son, J. E. & Lee, J. M. Light-controlled fruit
508 pigmentation and flavor volatiles in tomato and bell pepper. *Antioxidants* **9**, 14
509 (2019).
510 24. Liu, T. et al. Improved peach peel color development by fruit bagging. Enhanced
511 expression of anthocyanin biosynthetic and regulatory genes using white non-
512 woven polypropylene as replacement for yellow paper. *Sci. Hortic.* **184**, 142-148

(2015).

25. Lado, J. et al. Light regulation of carotenoid biosynthesis in the peel of mandarin and sweet orange fruits. *Front Plant Sci.* **10**, 1288 (2019).

26. Hu, L., Yang, C., Zhang, L., Feng, J. & Xi, W. Effect of light-emitting diodes and ultraviolet irradiation on the soluble sugar, organic acid, and carotenoid content of postharvest sweet oranges (*Citrus sinensis* (L.) Osbeck). *Molecules* **24**, 3440 (2019).

27. Liu, L. H., Zabaras, D., Bennett, L. E., Aguas, P. & Woonton, B.W. 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**, 495-500 (2009).

28. Ma, G. et al. Effect of blue and red LED light irradiation on beta-cryptoxanthin accumulation in the flavedo of citrus fruits. *J. Agric. Food Chem*. **60**, 197-201 (2012).

29. Llorente, B., Martinez-Garcia, J. F., Stange, C. & Rodriguez-Concepcion, M., Illuminating colors: regulation of carotenoid biosynthesis and accumulation by light. *Curr. Opin. Plant Biol.* **37**, 49-55 (2017).

30. Cronje, P. J. R., Barry, G. H. & Huysamer, M. Canopy position affects pigment expression and accumulation of flavedo carbohydrates of 'Nules Clementine' mandarin fruit, thereby affecting rind condition. *J. Am. Soc. Hortic. Sci.* **138**, 217–224 (2013).

31. Kondo, S. et al. Abscisic acid metabolism and anthocyanin synthesis in grape skin are affected by light emitting diode (LED) irradiation at night. *J. Plant Physiol.*

171, 823-829 (2014).

32. Zhang, L. et al. Regulation of carotenoid accumulation and the expression of carotenoid metabolic genes in citrus juice sacs in vitro. *J. Exp. Bot.* **63**, 871-886 (2012).

33. Lintig, J. V. et al. Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. *Plant J.* **12**, 625–634 (1997).

34. Toledo-Ortiz, G., Huq, E. & Rodriguez-Concepcion, M. Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 11626-11631 (2010).

35. Xie, B. X.et al. Supplemental blue and red light promote lycopene synthesis in tomato fruits. *J. Integr. Agric.* 18, 590-598 (2019).

36. Rosado, D., Trench, B., Bianchetti, R. E., Zuccarelli, R. & Rossi, M. Downregulation of phytochrome-interacting factor 4 influences plant development and fruit production. *Plant physiol.* **181**, 1360-1370 (2019).

37. Fujisawa, M., Nakano, T., Shima, Y. & Ito, Y. A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. *Plant Cell* **25**, 371-386 (2013).

38. Fujisawa, M. et al. Transcriptional Regulation of fruit ripening by tomato FRUITFULL homologs and associated MADS box proteins. *Plant Cell* **26**, 89-101 (2014).

39. Wang, X. et al. PpERF3 positively regulates ABA biosynthesis by activating

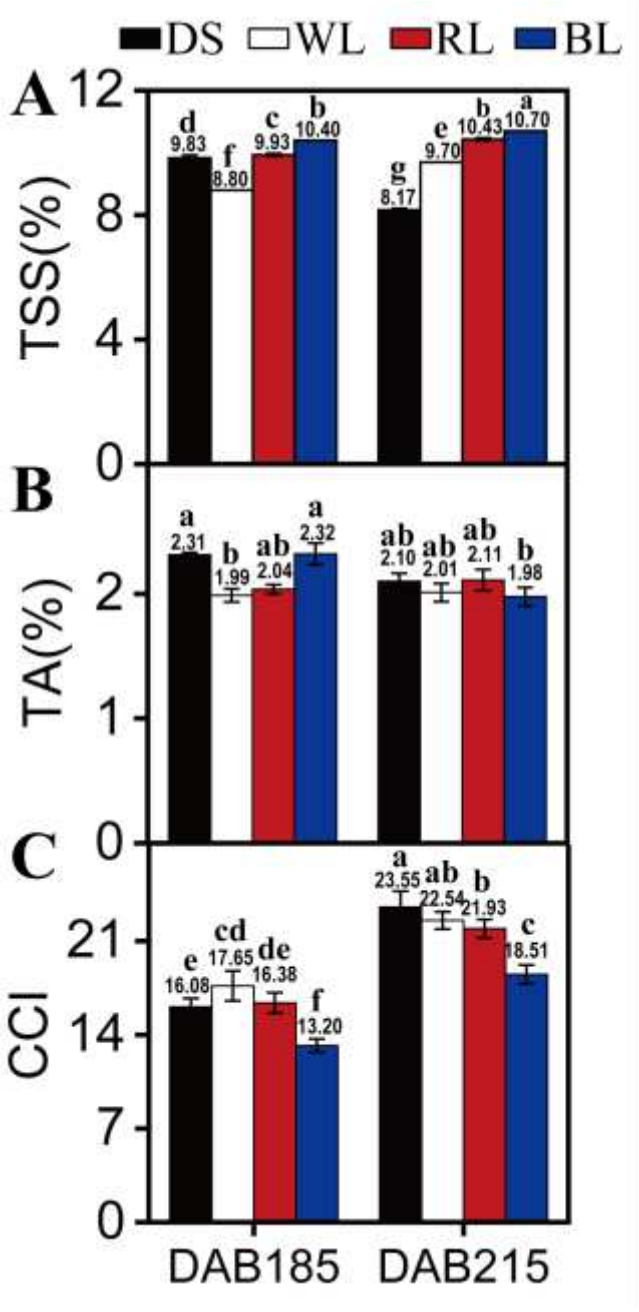
PpNCED2/3 transcription during fruit ripening in peach. *Hortic Res.* **6**, 19 (2019).

40. Welsch, R., Maass, D., Voegel, T., DellaPenna, D. & Beyer, P. Transcription factor RAP2.2 and its interacting partner SINAT2: stable elements in the carotenogenesis of Arabidopsis leaves. *Plant Physiol.* **145**, 1073–1085 (2007).
41. Mohanty, B. et al. Light-specific transcriptional regulation of the accumulation of carotenoids and phenolic compounds in rice leaves. *Plant Signal Behav.* **11**, 3002-3020 (2016).
42. Kou, X. et al. SNAC4 and SNAC9 transcription factors show contrasting effects on tomato carotenoids biosynthesis and softening. *Postharvest Biol. Technol.* **144**, 9-19 (2018).
43. Zhu, M., et al. A new tomato NAC (NAM/ATAF1/2/CUC2) transcription factor, SlNAC4, functions as a positive regulator of fruit ripening and carotenoid accumulation. *Plant Cell Physiol.* **55**, 119-135 (2014).
44. Ma, N. et al. Overexpression of tomato SlNAC1 transcription factor alters fruit pigmentation and softening. *BMC Plant Biol.* 14, 351 (2014).
45. Wang, Z., Rashotte, A. M. & Dane, F. *Citrullus colocynthis* NAC transcription factors CcNAC1 and CcNAC2 are involved in light and auxin signaling. *Plant Cell Rep.* 33, 1673-1686 (2014).
46. Gong, J. et al. Red light-induced kumquat fruit colouration is attributable to increased carotenoid metabolism regulated by FcrNAC22. *J. Exp. Bot.* erab283 (2021).
47. Han, Y. et al. Characterization of OfWRKY3, a transcription factor that positively

regulates the carotenoid cleavage dioxygenase gene *OfCCD4* in *Osmanthus fragrans*. *Plant Mol. Biol.* **91**, 485-496 (2016).

48. Zheng, H., Zhang, Q., Quan, J., Zheng, Q. & Xi, W. Determination of sugars, organic acids, aroma components, and carotenoids in grapefruit pulps. *Food Chem.* **205**, 112-121 (2016).
49. Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357-360 (2015).
50. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).
51. Zhang, Q. et al. Transcriptional regulatory networks controlling taste and aroma quality of apricot (*Prunus armeniaca* L.) fruit during ripening. *BMC Genom.* **20**, 45 (2019).
52. Langfelder, P. & Horvath, S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9,** 559 (2008).

Figures and figure legends



595

596 **Fig. 1** Effect of grapefruit bagged with four light-transmitting bags on the TSS content
597 (A), TA content (B) and CCI (C) of grapefruits during ripening. Error bar indicate
598 standard error from three biological replicates (n = 3). DS: dark-shade treatment; RL:
599 red-light treatment; BL: blue-light treatment; WL: white-light treatment. Different
600 letters indicate statistically significant difference in one-way ANOVA analysis.

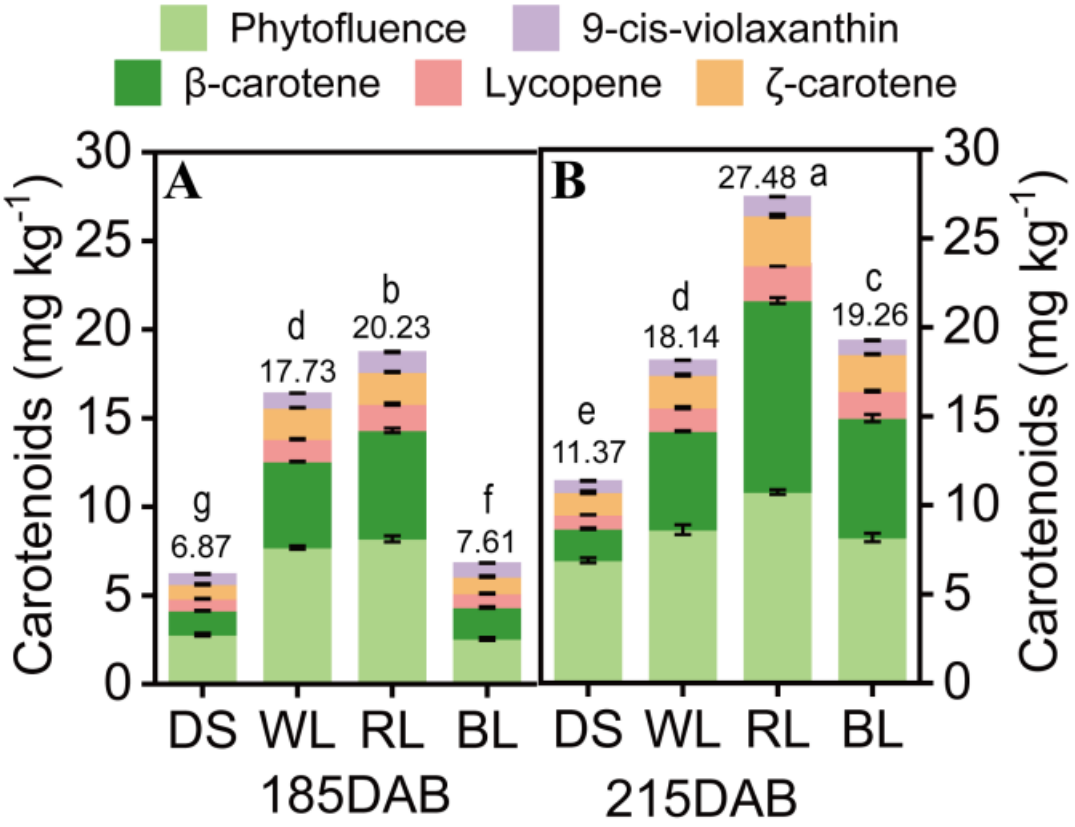
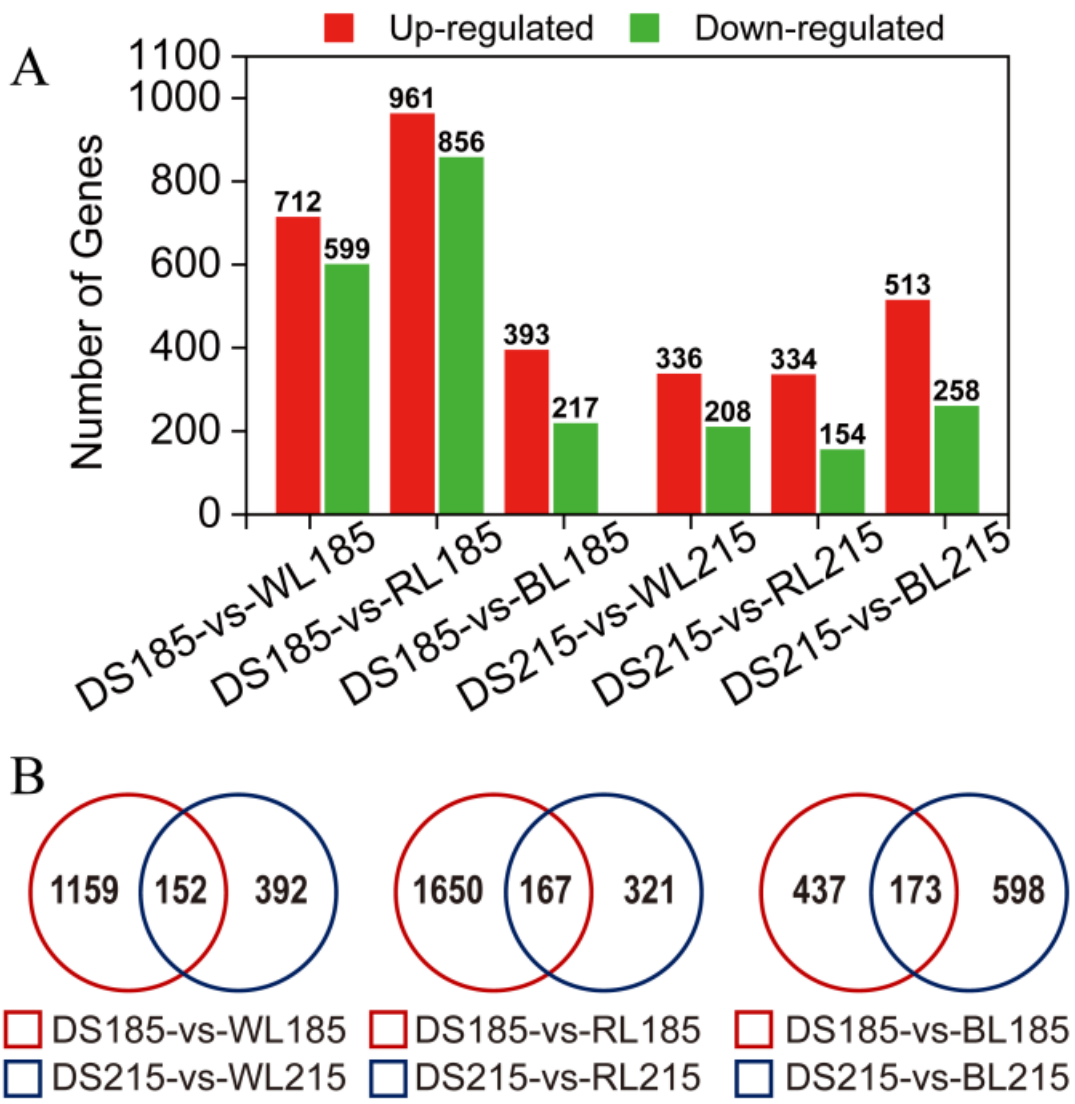


Fig. 2 Effects of different light treatments on content of carotenoid, including β-carotene, phytofluene, ζ-carotene, lycopene, and 9-*cis*-violaxanthin content during grapefruit ripening. Error bars indicate the standard error from three biological replicates (n = 3). DS: dark-shade treatment; RL: red-light treatment; BL: blue-light treatment; WL: white-light treatment. Different letters indicate statistically significant difference in one-way ANOVA analysis.



608

609 **Fig. 3** Profiling the changed genes and DEGs between grapefruit at different ripening

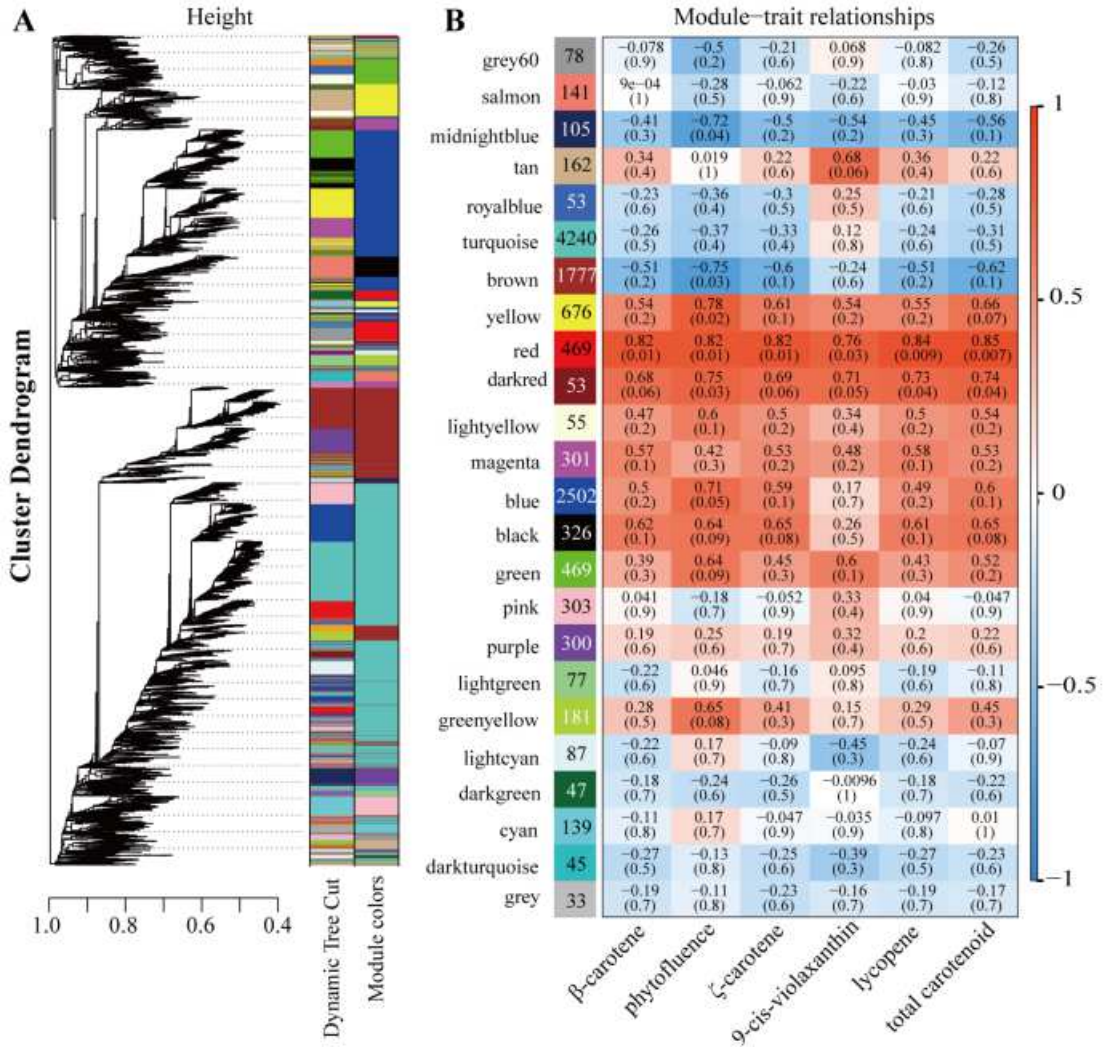
610 stages. (A) The histogram presents the number of upregulated and downregulated genes

611 between samples during grapefruit ripening. (B) Venn diagram for DEGs between

612 grapefruit samples at two ripening stages. “A” is the control group and “B” was the

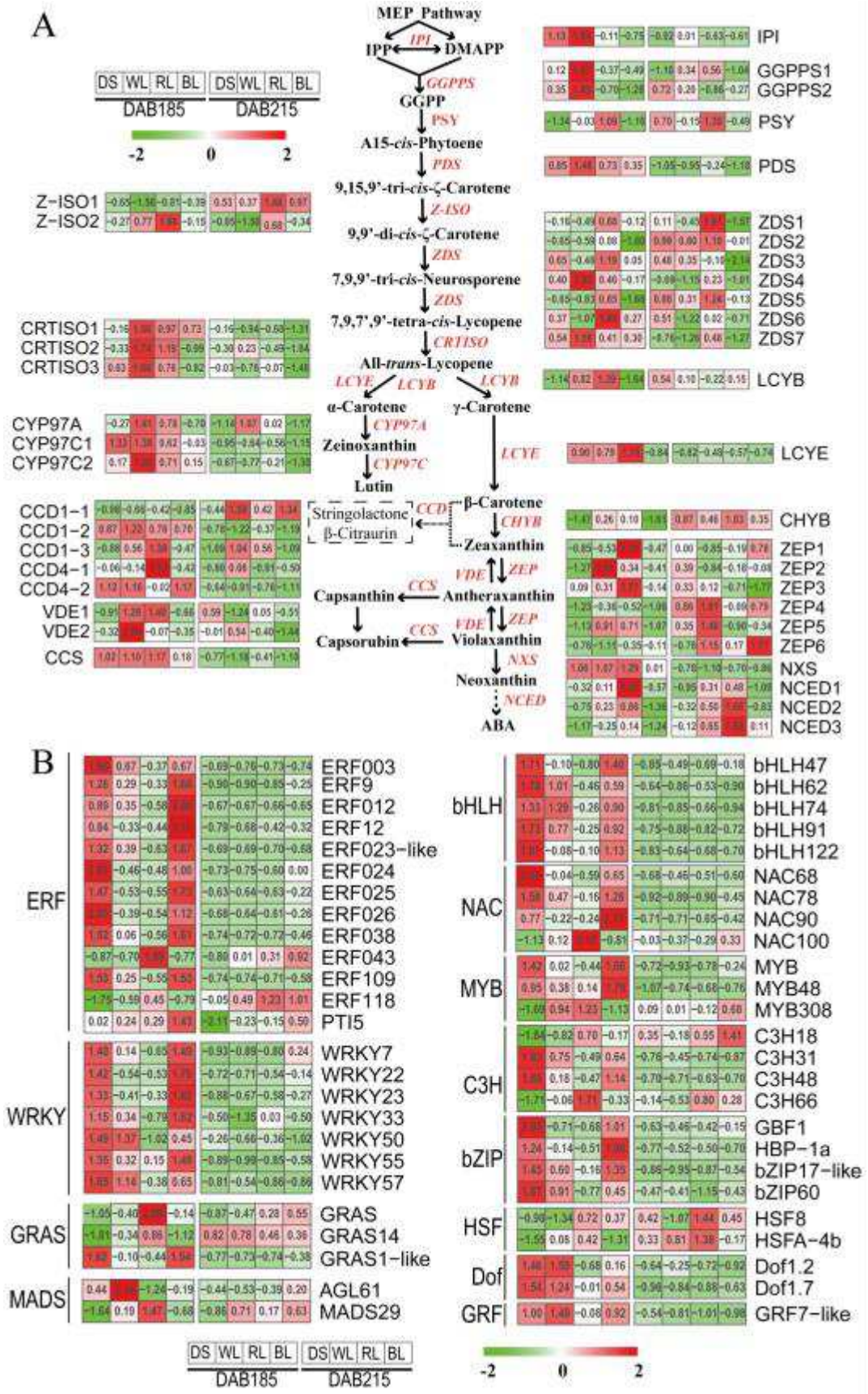
613 treatment group in “A-vs-B”. DS: dark-shade treatment; RL: red-light treatment; BL:

614 blue-light treatment; WL: white-light treatment.



615

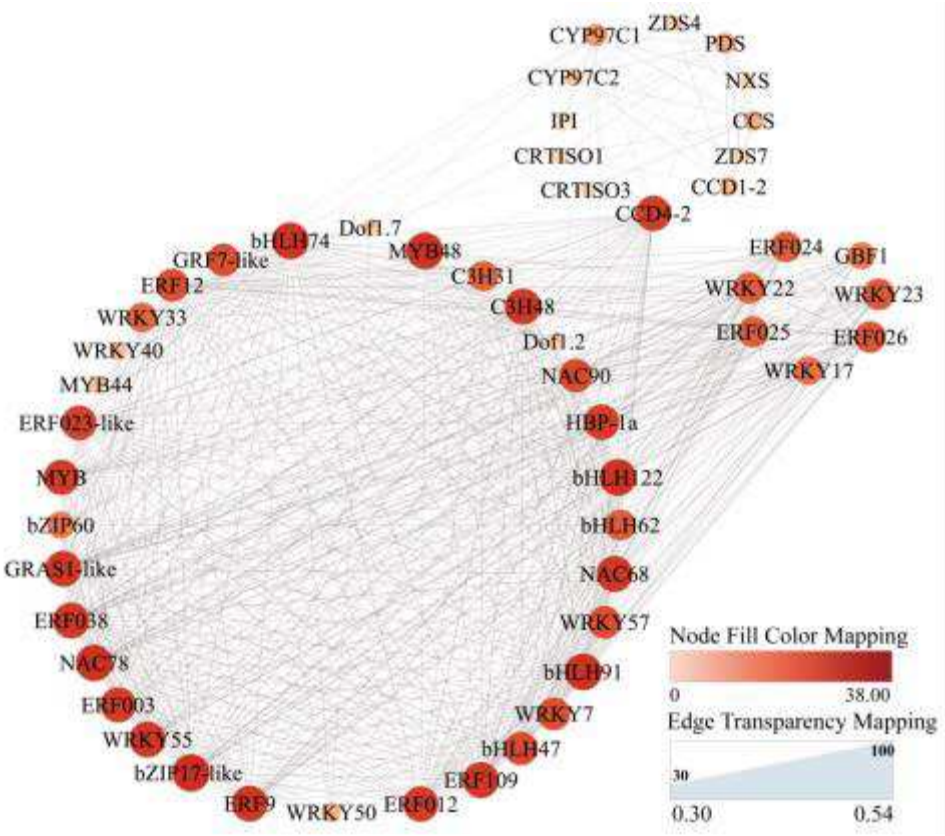
616 **Fig. 4** Weighted gene co-expression network analysis of grapefruit during ripening
617 under different light-transmittance treatments. A. Hierarchical clustering tree displays
618 twenty-four modules of co-expressed genes, in which each leaf represents one gene. B.
619 Modules related to carotenoid and corresponding *p*-values. The left panel indicates
620 twenty-four modules and the number of genes contained by each module. The right
621 panel displays a color scale for module or trait correlations from −1 to 1.



622

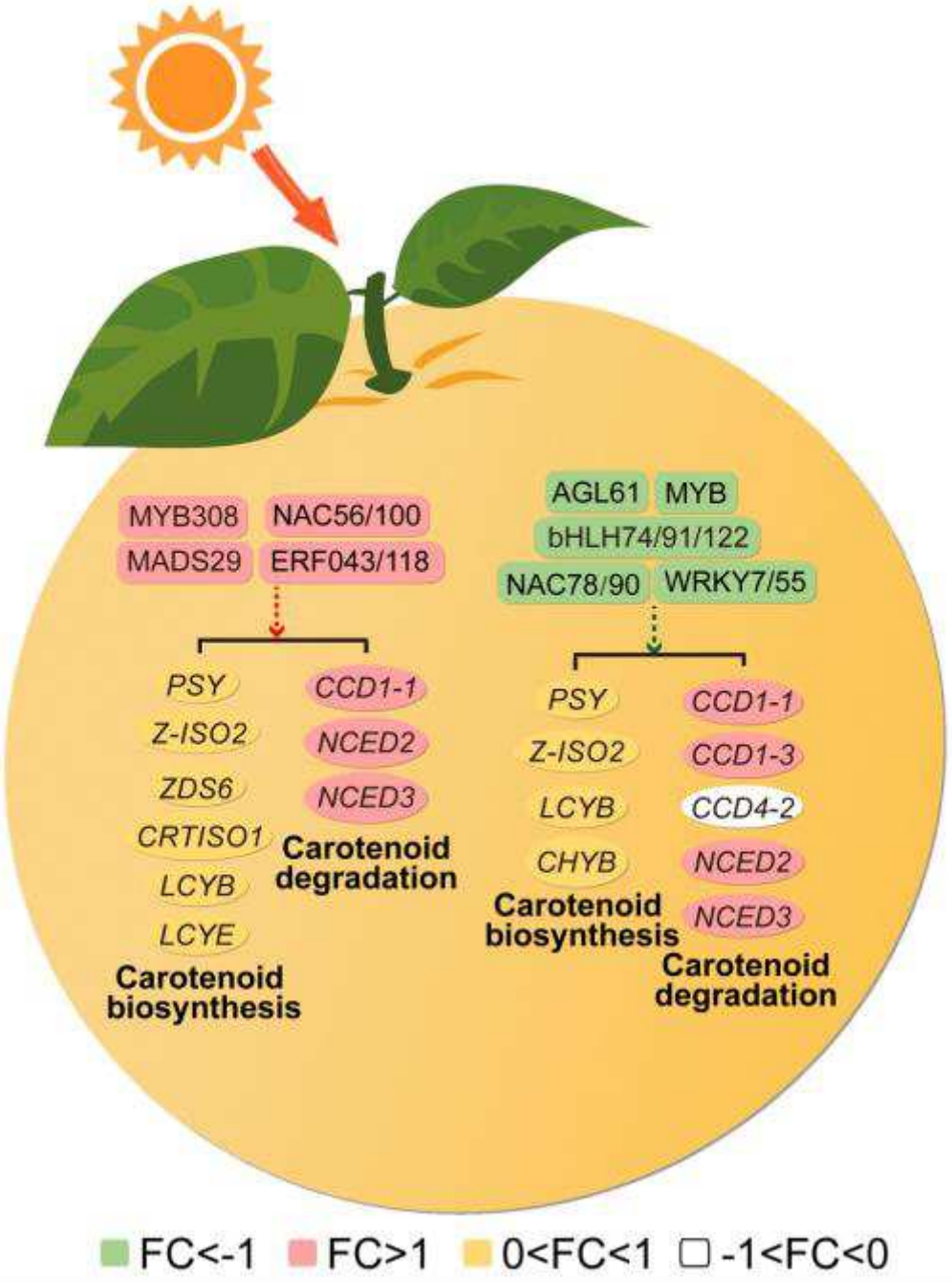
623 **Fig. 5** Heatmap analysis of structural genes (A) and transcriptional facors (B) correlated

624 with carotenoid metabolism during grapefruit ripening. Rows and columns indicate
625 gene names and samples in the heatmap, respectively. Red, white and green represent
626 high, medium and low expression level for genes. DS: dark-shade treatment; RL: red-
627 light treatment; BL: blue-light treatment; WL: white-light treatment.



628

629 **Fig. 6** The co-expression network of TFs and structural genes related to carotenoid
630 metabolism. Dot sizes and colors represent the numbers for correlated genes.



631

632 **Fig. 7** The proposed models of carotenoid metabolism mediated by red light during
633 grapefruit ripening. Pale pink and light green rounded rectangle, respectively,
634 represents up- and down-regulated TFs in response to RL. Red and green arrow indicate
635 positive and negative regulation of TFs on structural genes, respectively. Oval suggests

636 structural genes in carotenoid metabolic pathway. Different colors represent up- and
637 down-regulation level for genes in response to RL.
638

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplemetaryfigures.pdf
- Supplemetarytables.docx