**Original Article**

# Effects of light quality, photoperiod, $CO_2$ concentration, and air temperature on chlorogenic acid and rutin accumulation in young lettuce plants

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**Abstract**

Environmental stimuli modulate plant metabolite accumulation, facilitating adaptation to stressful conditions. In this study, the effects of blue and red light, photoperiod, $CO_2$ concentration, and air temperature on the chlorogenic acid (CGA) and rutin contents of lettuce (*Lactuca sativa* L.) were evaluated. Under continuous blue light and a high $CO_2$ concentration (1,000 ppm), the CGA level increased. The increased expression of *phenylalanine ammonia-lyase* (*PAL*) and activity of its product were correlated with high expression of *cinnamate 4-hydroxylase* (*C4H*) and *coumarate 3-hydroxylase* (*C3H*). Furthermore, changes in PAL activity altered the CGA content in lettuce exposed to the three environmental factors, blue light, continuous lighting and high $CO_2$ concentration. In addition, the expression levels of genes related to flavonoid biosynthesis increased in accordance with the promotion of CGA accumulation by the environmental factors. Under continuous blue light, 400 ppm $CO_2$ promoted rutin accumulation to a greater degree compared to 1,000 ppm $CO_2$, by downregulating *DFR* expression. Low air temperature induced CGA accumulation in lettuce grown under continuous blue light and 1,000 ppm $CO_2$. Therefore, light quality, photoperiod, $CO_2$ concentration, and air temperature exert synergistic effects on the CGA and rutin contents of lettuce by modulating activity in the corresponding biosynthesis pathways.

**Keywords**: flavonoids, phenolic compounds, phenylpropanoid pathway, polyphenol, polyphenol

oxidase

## 1 Introduction

Antioxidants, such as phenolic compounds and ascorbic acid, not only remove reactive oxygen species (ROS) in plants but also exert anti-inflammatory and anticancer effects against human pathologies (Heinonen *et al*., 1998; Wang and Jiao, 2000; Kumar and Pandey, 2013). Polyphenols in fruits and vegetables, such as phenolic acids, flavonols, anthocyanidins, have health-promoting effects (Scalbert and Williamson, 2000).

Environmental conditions alter the metabolite contents of plants. For example, salinity stress increases the sugar content of tomato fruit (Saito *et al*., 2008). Also, Ntagkas *et al*. (2016) showed that direct red-light irradiation of tomato fruit increased the ascorbic acid content. In the case of some leafy vegetables, spinach, potherb mustard, turnip leaf and so on, drought stress increased up the ascorbic acid and polyphenol content (Koyama et al., 2012). Furthermore, light conditions, light quality, photoperiod, and light intensity increase the antioxidant content in leaf of lettuce (Johkan *et al*., 2010; Oh *et al*., 2009) and *Arabidopsis* (Smirnoff, 2000). Regarding light quality, the quercetin and *flavonol synthase* (*FLS*) expression in lettuce were increased by UV-B and blue light (Ebisawa *et al*., 2008). Pigmentation of fruits and plant bodies is influenced by light quality; for example, in tomato fruit the redness of epidermal tissue results from carotenoid and lycopene.

55 Phytoene synthase is a key mediator of the synthesis of these pigments, and its activity is regulated
56 by the quality of irradiated light, with red light promoting such activity via PHY signaling (Schofield
57 and Paliyath, 2005). In coriander; the optimal ratio of red to blue light resulted in significant
58 antioxidant accumulation (Naznin *et al*., 2016). Oh *et al*. (2009) reported that heat shock, chilling,
59 and high light intensity significantly increase antioxidant activity in lettuce. The phenol and
60 flavonoid contents of ginger vary with $CO_2$ concentration (Ghasemzadeh *et al*., 2010). In addition,
61 drought stress modulates the production of phenylpropanoids and carotenoids in grape (Savoi *et al*.,
62 2016).

63 Chlorogenic acid (CGA) is a polyphenol implicated in enzymatic browning in lettuce
64 (Altunkaya and Gokmen, 2008). However, polyphenols such as CGA are strong antioxidants that
65 reduce oxidative damage in human cells (Khanam *et al*., 2012). The CGA content of lettuce was
66 greatly increased by certain combinations of environmental factors (Yoshida *et al*., 2016, Shimomura
67 *et al*., 2020). The CGA content was affected by the irradiance level and light quality of overnight
68 supplemental lighting and $CO_2$ enrichment, but no effects on the other bioactive compounds, such
69 anthocyanin and carotenoid. Environmental control technology could therefore be used to control the
70 phytochemical contents of plants (Yoshida *et al*., 2016). Furthermore, the combination of continuous
71 blue light and a high $CO_2$ concentration increased the CGA content of young-leaf lettuce
72 (Shimomura *et al*., 2020). Blue light increased the contents of polyphenols, including CGA, in

73 lettuce (Johkan *et al*., 2010). Furthermore, continuous light increased the antioxidant levels in lettuce
74 (Bian *et al*., 2016). Moreover, environmental factors such as drought stress can increase the levels of
75 antioxidants, such as ascorbic acid and polyphenols (Koyama *et al*., 2012), whereas salinity stress in
76 hydroponics increases CGA accumulation in honeysuckle (Yan *et al*., 2016). In a red-pigmented
77 lettuce, mild light stress and an elevated $CO_2$ concentration resulted in the accumulation of phenolics
78 and increased antioxidant activity (Pérez-López *et al*., 2018). Therefore, controlled conditions, such
79 as in a plant factory, could be used to modulate the phytochemical compositions of leafy vegetables.
80 However, the mechanisms by which combinations of environmental factors affect metabolic
81 pathways related to phenolic compounds in lettuce are unclear. From our previous reports (Yoshida
82 *et al*., 2016, Shimomura *et al*., 2020), we have a hypothesis that a specific combination of multi
83 environmental factors could induce the great accumulation of CGA in phenylpropanoid pathway,
84 through the activation of some specific gene expressions, furthermore, it can also affect to other
85 compounds included in flavonoid pathway.

86 In this study, we assessed the effects of environmental factors (light quality, photoperiod,
87 and $CO_2$ concentration) on the CGA and rutin, as one of the flavonoids, content of lettuce.
88 *Phenylalanine ammonia-lyase* (*PAL*) expression and PAL activity were regulated by light quality,
89 photoperiod, and $CO_2$ concentration, and the increased *PAL* activity resulted in an increased CGA
90 content in lettuce. In addition, there were cross effects of different environmental factors on gene

91 expressions and PAL activity, a certain combination of environmental factors induced the synergic
92 increase of CGA content and related gene expressions, PAL activity. Furthermore, low air
93 temperature promoted CGA accumulation under continuous blue light and a high $CO_2$ concentration.
94 The expression levels of genes related to flavonoid biosynthesis were upregulated under continuous
95 blue light and a high $CO_2$ concentration. However, specifically, a low $CO_2$ concentration increased
96 the rutin content by downregulating *dihydroflavonol 4-reductase* (*DFR*) expression.

97 **2 Materials and Methods**

98 *2.1 Plant materials*

99 Seeds of green leaf lettuce (*Lactuca sativa* L., cv. Green Wave; Takii Seed Co., Ltd., Kyoto,
100 Japan) were germinated on urethane sponges dipped in half-strength commercial nutrient solution
101 (Otsuka A; OAT agrio Co., Ltd., Tokyo, Japan). Seeds were grown at 25°C in a growth chamber
102 (LPH-411SPC; Nippon Medical & Chemical Instruments, Co., Ltd., Osaka, Japan) for 2 weeks at a
103 photosynthetic photon flux density (PPFD) of 100 μmol $m^{-2}$ $s^{-1}$ from white fluorescent lamps (FL,
104 FLR40S・EX-W/M/36; Mitsubishi Electric Lighting Co., Ltd., Kamakura. Japan). PPFD was
105 measured using a light quantum sensor (LI-190A,; Li-Cor, Inc., Lincoln, NE, USA). The wavelength
106 of the light source was determined using a spectroradiometer (USB2000; Ocean Optics, Dunedin, FL,
107 USA). The photoperiod was set at 12 h day/12 h night.

108 *2.2 Cultivation and experimental growth conditions*

After 2 weeks, seedlings were transplanted to predetermined treatment conditions. Experiments were conducted in a growth cabinet (CFH-415; Tomy Seiko Co., Ltd., Tokyo, Japan).

*2.2.1 Transcriptomic analysis of lettuce seedlings grown under continuous blue light and a high $CO_2$ concentration*

Plants were grown at a PPFD of 200 μmol $m^{-2}$ $s^{-1}$. The atmospheric conditions were as follows: $CO_2$ concentration, 1,000 ppm; air temperature, 25°C; and relative humidity, 70%. The light sources were a red LED (peak wavelength: 650 nm; ISL-150×150-RR; CCS, Inc., Kyoto, Japan), and blue LED (B; peak wavelength: 465 nm; ISL-150×150-BB; CCS, Inc.). Lettuce seedlings were transferred to a Petri dish containing the abovementioned commercial nutrient solution. The surface of the Petri dish was wrapped with aluminum foil, and six plants were grown under each of the three experimental conditions. Two days after transplantation, three plant shoots (including the apex) were harvested from each light source as three biological replicates and ground in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with DNase using the RNase-free DNase Set (Qiagen). After performing quality control of total RNA using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), qualified samples were used for library construction. RNA-seq was conducted using the outsourcing server of TaKaRa Co., Ltd. (Shiga, Japan). Strand-specific libraries were constructed using the TruSeq Stranded RNA Library Prep Kit and sequenced on a NovaSeq 6000 system (Illumina, San Diego, CA, USA) in 100

127 bp paired-end mode. The raw RNA-seq reads were processed to remove adaptors and low-quality
128 sequences using a custom Perl script combined with the FASTX-toolkit
129 (http://hannonlab.cshl.edu/fastx_toolkit/), and aligned to a lettuce genome (Lettuce Genome
130 Consortium, UC Davis Genome Center; https://lgr.genomecenter.ucdavis.edu/Home.php;
131 Reyes-Chin-Wo *et al*., 2017) using TopHat2 (Kim *et al*. 2013;
132 http://tophat.cbcb.umd.edu/downloads/tophat-2.0.11.Linux_x86_64.tar.gz). After genome alignment,
133 gene expression levels were evaluated using Cuffdiff (Trapnell *et al*. 2013). Lettuce protein
134 sequences were subjected to InterProscan v4 to obtain GO and InterPro ID information. BLASTp
135 was also performed by using *Arabidopsis thaliana* TAIR10 gene set as database to estimate the
136 functions of lettuce genes. These information are provided as Supplementary data 1.
137 *2.2.2 Effects of light quality, photoperiod, and $CO_2$ concentration on CGA and rutin contents, and*
138 *gene expression*
139 Under continuous light or a 12 h light/12 h dark photoperiod, at 25°C and 70% relative
140 humidity, a red LED (peak wavelength, 650 nm; ISL-150×150-RR; CCS, Inc.) or blue LED (peak
141 wavelength, 465 nm; ISL-150×150-BB; CCS, Inc.) (Fig. 1) was set in a growth chamber. PPFD was
142 adjusted to 200 μmol $m^{-2}$ $s^{-1}$ around the plant canopy. The $CO_2$ concentration was set to 400 ppm
143 (low) or 1,000 ppm (high). In our previous study (Shimomura *et al.*, 2020), we found the most
144 effective levels of each environmental factors, light quality, photoperiod, light intensity and $CO_2$

concentration, on CGA content in young lettuce plants. Based on our previous results, we selected those combinations of environmental factors and levels in this experiment. Treatments were assigned by the randomized complete block method. Growth chambers continuously illuminated by red and blue LEDs were set to the appropriate $CO_2$ levels. When they showed four to five true leaves, six seedlings were transferred to a growth chamber under continuous blue or red light. In subsequent experiments, we used a 12 h photoperiod together with the above described levels of the other environmental factors. Seedlings were transferred to a Petri dish containing the abovementioned commercial nutrient solution. The Petri dish was wrapped with aluminum foil. Two days after treatment, plant shoots were harvested, and we transferred a 0.5 g sample from the middle of a true leaf to a microtube. The samples were snap-frozen in liquid nitrogen and stored in a freezer at -80°C. The sampled leaves were ground in 1 mL of methanol using the Tissue Lyser II instrument (Qiagen) and lyophilized.

*2.2.3 Effects of air temperature on CGA content and the expression of genes related to phenylpropanoid and flavonoid biosynthesis*

When they had four to five true leaves, six seedlings were transferred to growth chambers at 15, 20, and 25°C under continuous blue LED light and 1,000 ppm $CO_2$. PPFD was adjusted to 200 μmol $m^{-2}$ $s^{-1}$ around the plant canopy, and the seedlings were transferred to a Petri dish. Two days after treatment, plant shoots were harvested, and we transferred a 0.5 g sample from the middle of a

163 true leaf to a microtube.

164 *2.3 Evaluation of plant growth and CGA content*

165 Shoot fresh weight (FW) and dry weight (DW) were measured at the time of harvesting.
166 We transferred a 0.5 g sample from the middle of a true leaf to a microtube. The samples were
167 snap-frozen in liquid nitrogen and stored in a freezer at -80℃. The samples were ground in 1 mL of
168 methanol using the Tissue Lyser II instrument (Qiagen), and centrifuged for 5 min (9,000 ×g); the
169 supernatants were passed through a 2-μm membrane filter. The supernatants were then diluted
170 twofold with dilution buffer (methanol: distilled water: formic acid 70: 426: 4). CGA content was
171 determined using a high-performance liquid chromatography (HPLC) system (GL7400 [PDA
172 detector, GL-7452]; GL-Sciences, Inc., Tokyo, Japan) and HPLC column (Inertsil ODS-SP, 5 μm,
173 3.0 × 150 mm; GL-Sciences, Inc.). The column temperature was maintained at 40℃. The mobile
174 phase consisted of buffers A (methanol) and B (5 mM $KH_2PO_4$, pH 2.5) at a flow rate of 0.4 mL
175 $min^{-1}$. The detection wavelength was 280 nm. For rutin, the mobile phase consisted of buffers A
176 (methanol), B (tetrahydrofuran), and C (20 mM $KH_2PO_4$, pH 3.0), at a 3:2:5 ratio and flow rate of
177 1.0 mL $min^{-1}$.

178 2.4 *Gene expression analysis*

179 Total RNA was extracted from plant-leaf samples with the RNeasy Plant Mini Kit
180 (Qiagen) and used as the template for cDNA synthesis with the PrimeScript II First-Strand cDNA

Synthesis Kit (TaKaRa). Real-time quantitative PCR analysis of the expression of genes related to phenylpropanoid and flavonoid synthesis, and the housekeeping gene *ACTIN*, was performed using Brilliant II SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). The primers are shown in supplementary material table 1. Quantitative PCR was performed using the Mx3000p instrument (Stratagene). Three biological samples were analyzed per treatment, each in triplicate. Gene expression was normalized to that of *ACTIN* and is presented as a ΔCt value.

*2.4 PAL activity assay*

Leaf samples were ground in 1 mL of methanol using the Tissue Lyser II instrument (Qiagen), and 1 mL of extraction buffer (50 mM Tris-HCl [pH 8.8], 10 mM 2-mercaptoethanol, 1 mM EDTA, and 2.5% PVP-40) was added to 0.1 g of sample and incubated at 4°C for 1 hour. Next, the samples were centrifuged for 20 min (9,000 × g), and the supernatants were passed through a 2-µm membrane filter. Filtered supernatants were desalted by centrifugation at 5,000 × g for 30 min using a Centrifugal Filter (Amicon Ultra-15; Merck, Japan, Co., Ltd., Tokyo, Japan). Tris-HCl buffer (100 mM) was used as the diluent. Each well of a 96-well microplate contained 50 µL of diluted sample, 100 µL of reaction solution (100 mM Tris-HCl, pH 8.8), and 50 µL of substrate [40 mM L-phenylalanine (≥ 98%; Sigma-Aldrich, St. Louis, MO, USA) and 100 mM Tris-HCl, pH 8.8], which were mixed and incubated at 37°C for 30 min. The reaction was terminated by adding 12 µL of 4M HCl. The amount of *t*-cinnamic acid equivalent was measured at a wavelength of 290 nm

using a microplate spectrophotometer (Multiskan GO; Thermo Fisher Scientific K.K., Yokohama, Japan). A calibration curve was prepared using *t*-cinnamic acid (≥ 99%; Sigma-Aldrich). The protein concentration was measured by the Bradford method using the Bradford Protein Assay Kit (TaKaRa). A standard calibration curve was created using BSA standard solutions. Samples (4 μL) were dispensed into wells of a 96-well microplate, and 200 μL of Bradford Dye Reagent was added, mixed, and incubated at 25°C for 5 min. Deionized water (200 μL) was added to the blank wells. Absorbance at 595 nm was determined using the Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific K.K.). One unit of PAL activity is defined as the amount of enzyme that produce one nano mole of cinnamic acid for one hour 37 ℃.

*2.5 Statistical analysis*

After confirming data normality using the Ryan–Joiner test, and homoscedasticity by Levene's test, analysis of variance (ANOVA) was carried out in general linear model (glm) mode using R software (version 3.6.1; https://cran.r-project.org). To compare means, Tukey's *post hoc* test was performed using R software. *P*-values ≤ 0.05 were considered significant.

## 3. Results

*3.1 Transcriptomic analysis of lettuce seedlings grown under continuous blue light and a high $CO_2$ concentration*

Significant changes in the expression of 192 genes were detected in lettuce cultivated

under continuous blue and red light and a high $CO_2$ concentration ($p < 0.05$, $|\log_2$ fold-change (fc)$| > 2$). The expression of several genes related to polyphenol biosynthesis increased significantly under blue light (Table 1). The expression levels of one *phenylalanine ammonia-lyase* (*PAL(2)*), one *chalcone synthase* (*CHS(2)*), one *chalcone isomerase* (*CHI(2)*), one *flavanone 3-hydroxylase* (*F3H*) and one *flavonol synthase* (*FLS*) were increased under blue light. Furthermore, it tended that the expression levels of the expression of one *phenylalanine ammonia-lyase* (*PAL(1)*), one *cinnamate 4-hydroxylase* (*C4H*), one *4-coumarate-CoA ligase* (*4CL*), one *chalcone synthase* (*CHS(1)*), two *chalcone isomerase* (*CHI(1), (3)*), one *dihydroflavonol 4-reducatase* (*DFR*), one *anthocyanidin synthase* (*ANS*), one *coumarate 3-hydroxylase* (*C3H*) were increased under blue LED. By contrast, there was a tendency that the expression levels of two *MYB90/PAP2* and one *KNOX* gene were increased under red light.

*3.2 Effects of light quality, photoperiod, and $CO_2$ concentration on CGA and rutin contents*

The CGA content of young lettuce plants was affected by the environmental factors (Fig. 2). The CGA content per 100 g FW was significantly increased to > 200 mg/100g under continuous blue light and a high $CO_2$ concentration. Furthermore, 24 h of blue light increased the CGA content twofold compared to red light. By contrast, the CGA content per gram DW was increased by blue light with a 24 h photoperiod. There were no interaction effects.

Only 24 h of blue light with 400 ppm $CO_2$ significantly increased (to 100 mg/100g FW) the

235 rutin content of young lettuce plants (Fig. 2); the other treatments yielded a rutin content < 50
236 mg/100g FW. The rutin content per gram DW tended to be lower in the presence of 1,000 compared
237 to 400 ppm $CO_2$.

238 *3.3 Effects of light quality, photoperiod, and $CO_2$ concentration on CGA and rutin biosynthesis gene*
239 *expression and PAL activity*

240 The expression levels of two *PAL*-like genes were influenced by the environmental factors.
241 Especially, *PAL(2)* expression under blue light and a high $CO_2$ concentration tended to be
242 significantly higher with a 24 than 12 h photoperiod (Table 2). *C4H* expression was significantly
243 increased by blue light. $CO_2$ concentration affected *C4H* expression under blue, but not red, light.
244 *4CL* expression was not affected by any of the environmental factors. *C3H* expression tended to be
245 higher at 1,000 than 400 ppm $CO_2$. The expression of *CHS*, *CHI(2)*, *F3H*, and *FLS*, which are
246 related to flavonoid biosynthesis, was higher under blue than red light; only the expression of *CHS*
247 was increased by continuous blue light. The expression of *DFR* tended to be promoted only by high
248 $CO_2$ conditions under both red and blue light.

249 PAL activity was affected by the environmental factors, including the combination of light
250 quality and $CO_2$ concentration (Fig. 3). PAL activity was significantly higher under blue than red
251 light. In addition, PAL activity was higher with a 24 h photoperiod, particularly under blue light.
252 Under blue light, PAL activity tended to be higher with 1,000 than 400 ppm $CO_2$. PAL activity was

253 twofold higher under continuous blue light and 1,000 ppm $CO_2$ than with a 24 h photoperiod under
254 blue or red light and 400 ppm $CO_2$.

255 *3.4 Effects of air temperature on CGA content and the expression of genes related to*
256 *phenylpropanoid and flavonoid biosynthesis*

257 Air temperature affected CGA content. Under continuous blue light and a high $CO_2$
258 concentration, the CGA content was significantly (66%) higher at 15°C than 25°C (Fig. 4). An air
259 temperature of 15°C increased the CGA content to 20 mg g $DW^{-1}$. Under red light, the CGA content
260 tended to increase as air temperature decreased.

261 Air temperature did not affect *PAL(1)* and *PAL(2)* expression (Table 3). The expression of
262 *C4H* was reduced at a low air temperature, but that of *4CL* and *C3H* was unchanged under
263 continuous blue light and a high $CO_2$ concentration. However, the expression of *CHS, F3H*, and
264 *CHI(2)*, which are related to flavonoid biosynthesis, was higher at 15°C. Indeed, *CHS* expression
265 was sevenfold higher at 15°C than 25°C. By contrast, the expression levels of *DFR*, *ANS*, and *FLS*
266 were not significantly influenced by air temperature.

267 **4. Discussion**

268 We previously reported (Shimomura *et al*., 2020) that continuous blue light at 200 μmol $m^{-2}$
269 $s^{-1}$ and $CO_2$ promoted the accumulation of CGA to > 200 mg per 100 g of fresh lettuce. Furthermore,
270 continuous lighting by fluorescent lamp and a high $CO_2$ concentration increased the CGA content. In

271 this study, especially, continuous and a high $CO_2$ concentration under blue light also increased the
272 CGA content of young lettuce than under red light. The rutin content was increased by continuous
273 blue light and a low $CO_2$ concentration (Fig. 2). Cvetic *et al*. (2009) reported that the total phenolic
274 content and antioxidant and peroxidase activities of moss increased under long-day conditions. This
275 indicates activation of a stress response under long-day conditions. Furthermore, in that study, the
276 phenolic compound composition was altered by modulating the $CO_2$ concentration and PPFD,
277 indicating that those factors affect the biosynthesis of phenolics.

278 In this study, blue light tended to upregulate the expression of *PAL*, *C4H*, *4CL*, *CHS*, *CHI*,
279 *F3H*, *DFR*, *ANS*, *C3H*, and *FLS* compared with red light (Table 1). The blue light receptor,
280 cryptochrome, induces blue light-dependent ROS synthesis (Consentiono *et al*., 2015). The increase
281 in nuclear ROS level mediated by cryptochrome triggers adaptation to environmental stresses in
282 *Arabidopsis* (El-Esawi *et al*., 2017). Environmental stresses may promote the production of phenolic
283 compounds by activating phenylpropanoid and flavonoid biosynthesis. Cryptochrome absorbs a
284 broad spectrum of light from UV to blue wavelengths. Kitazaki *et al*. (2018) reported that, in lettuce,
285 the expression levels of *PAL DFR*, and *CHS* were increased by blue light. Prohydrojasmon increases
286 the expression of several genes—such as *PAL*, *F3H*, and *ANS*—as well as CGA biosynthesis in
287 red-leaf lettuce (Takahashi *et al*., 2021). Blue light might also affect the expression of genes related
288 to phenolic compounds. In this study, the expression levels of several genes related to phenolics

289 biosynthesis were influenced not only by blue light, but also by the other environmental factors.
290 *PAL(2)* expression increased under continuous blue light and 1,000 ppm $CO_2$. Indeed, its expression
291 was modulated by light quality, photoperiod, and $CO_2$ concentration. Yan *et al*. (2016) reported that
292 salinity stress increased *PAL* expression and CGA content in honeysuckle. Furthermore, nitrogen
293 deficiency increases PAL activity and the accumulation of phenolic compounds (Mohanty *et al*.,
294 2016). Hartley *et al*. (2000) reported that, in some plant species, PAL activity is increased at elevated
295 atmospheric $CO_2$ concentrations. Oh *et al*. (2009) showed that PAL is important for environmental
296 stress tolerance and adaptation in lettuce. Blue light led to a greater increase in CGA content than red
297 light by upregulating *PAL* expression and PAL activity. *PAL(2)* expression increased in response to
298 the three environmental factors, light quality, photoperiod and $CO_2$ concentrations, but that of
299 *PAL(1)* was increased only by blue light. In *Arabidopsis*, *AtPAL* isogenes differentially respond to
300 environmental stresses (Zhang and Liu, 2015). We hypothesize that *PAL(2)* in lettuce are regulated
301 by different transcription factors and affected by different environmental factors. Furthermore, there
302 could be a synergic effect among different three environmental factors, light quality, photoperiod and
303 $CO_2$ concentrations on *PAL(2)* expression, because it greatly increased under continuous blue
304 lighting with high $CO_2$ concentration. Lakshmanan *et al*. (2015) suggested that the increased levels
305 of secondary metabolites under blue light were mediated by cryptochrome and the *WRKY* and *ZnF*
306 genes. Mohanty *et al*. (2016) reported that transcription factor genes, such as *ZnF*, *WRKY*, *MYB*, and

307 *ZIP*, are related to the biosynthesis of phenolic compounds in rice plants grown under stress
308 conditions (Mohanty *et al*., 2016). In *Taraxacum antungense*, the CGA content was increased by
309 upregulation of *HQT*, as mediated by the helix-loop-helix transcription factor *TabHLH1* (Liu *et al*.,
310 2021). *MYB*, *LIM*, and *KNOX* downregulate *PAL*, and *MYB* and *LIM* upregulate *PAL* (Zhang and Liu,
311 2015). By contrast, *KNOX* downregulates *PAL*. In this study, blue light inhibited *KNOX* expression
312 (Table 1). The relationship between *KNOX* expression and blue light is unknown, but upregulation of
313 *PAL* could be controlled by blue light via *KNOX*. Furthermore, transcription factors could mediate
314 the effects of photoperiod and $CO_2$ concentration on *PAL* expression. *MYB90(PAP2)* expressions was
315 affected by light quality. Bac-Molenaar *et al*. (2015) reported that natural variation of *MYB90*
316 *(PAP2)* caused anthocyanin accumulation in *Arabidopsis*. In summary, *MYB90 (PAP2)*-like genes
317 could be involved in the regulation of flavonoid biosynthesis in lettuce.

318 In this study, PAL activity was modulated by the environmental factors (Figs. 2 and 3). *C4H*
319 expression was upregulated by blue light and a high $CO_2$ concentration (Table 2). The increased PAL
320 and C4H activities under continuous blue light and a high $CO_2$ concentration likely resulted in
321 increased levels of cinnamic acid, *p*-coumaric acid, and *p*-coumaroyl CoA. In potato, increased *PAL*
322 expression was strongly correlated with increased levels of polyphenol compounds, including CGA
323 (André *et al*., 2009). Luna *et al*. (2016) reported that both CGA content and PAL activity increased
324 after cutting. The expression levels of *PAL(2)*, *C4H*, and *C3H* were increased at a high $CO_2$

325 concentration (Table 2). Ghasemzadeh *et al*. (2010) reported that the levels of flavonoids, phenolic
326 compounds, and antioxidants in ginger were increased at a high $CO_2$ concentration. Perez-Lopez *et*
327 *al*. (2018) showed that high light intensity and an elevated $CO_2$ concentration induced the production
328 of phenolics in lettuce. In this study, a high $CO_2$ concentration increased the CGA content by
329 activating phenylpropanoid biosynthesis pathways. In lettuce, a low $O_2$ concentration decreased PAL
330 activity (Ke and Saltveit, 1989). PAL activity may be linked to the increased CGA content (as
331 indicated by high *C4H* and *C3H* expression) seen in lettuce plants grown under continuous blue light
332 and a high $CO_2$ concentration. The expression of *PAL* and *C4H* increased simultaneously under
333 continuous blue light, and *PAL* and *C3H* expression was increased by a high $CO_2$ concentration. The
334 activation of *C3H* by a high $CO_2$ concentration may promote CGA accumulation in lettuce. The
335 expression of the stress responsive transcription factors, *MYB*, *DREB*, and *bZIP*, was affected by
336 $CO_2$ concentration (Palit *et al*., 2020). At a high $CO_2$ concentration, lettuce accumulated CGA,
337 caffeic and ferulic acid, quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide,
338 luteolin-7-*O*-glucoside, rutin, quercitrin, and kaempferol (Sgherri *et al*., 2017). Therefore, $CO_2$
339 concentration could induce stress responses in lettuce by modulating phenolics biosynthesis.

340 Low air temperature increased the CGA content of lettuce plants grown under continuous
341 blue light and a high $CO_2$ concentration (Fig. 4). The expression levels of *CHS*, *CHI(2), F3H*, and a
342 *DFR* (which are related to flavonoid biosynthesis) were significantly higher at 15°C than 25°C

343 (Table 3). A low air temperature results in a high anthocyanin level in lettuce (Gazula *et al*., 2005).
344 In kale, low air temperature increased the levels of phenolic compounds (Lee and Oh, 2015). In this
345 study, the expression levels of *PALs* and other genes related to phenylpropanoid biosynthesis, was
346 not increased at low temperature (Table 3). Simões *et al*. (2014) showed that PAL activity at 5°C
347 was higher than at 10°C in kale. Therefore, an air temperature of 15°C might not be sufficient to
348 stimulate *PAL* expression and PAL activity. Polyphenol oxidase (PPO) induces browning in freshly
349 cut lettuce (Luna *et al*., 2016). PPO converts CGA into quinones, which are substrates for the
350 synthesis of brown polymers. In potato, PPO activity peaks at around 40°C (Li *et al*., 2018). In
351 lettuce, the optimum temperature for PPO activity is 35°C (Gawlik-Dziki *et al*., 2008). It is possible
352 that, under continuous blue light and a high $CO_2$, concentration PPO activity was lower at 15°C than
353 at 25°C, thereby promoting CGA accumulation.

354 Under continuous blue light, *CHS* expression was activated, so the levels of CGA and
355 flavonoids including anthocyanidins would likely be increased. In red-leaf lettuce, pigments such as
356 anthocyanin are increased more by blue than by red light (Johkan *et al*., 2010). Blue and UV light
357 activate genes related to the phenylpropanoid pathway—such as *PAL*, *C4H*, *4CL*, *CHI*, *F3H*, *DFR*,
358 and *ANS*—and induce anthocyanin accumulation in tomato fruit (Kim *et al*., 2021). However, the
359 rutin content increased to 100 mg per 100 g FW only under continuous blue light and 400 ppm $CO_2$
360 (Fig. 2). Furthermore, rutin content tended to decrease at a high $CO_2$ concentration. Li *et al*. (2017)

reported that at an elevated $CO_2$ concentration, the expression levels of *PAL*, *C4H*, *4CL*, *CH*S, *CHI*, *F3H*, *DFR*, *ANS,* and *UFGT* were increased in green tea. In this study, *DFR* expression was higher at 1,000 compared to 400 ppm $CO_2$. A high $CO_2$ concentration could activate *DFR* and consume substrates for anthocyanin production instead of rutin, resulting in reduced rutin content.

Upregulation of *PAL* expression was correlated with high expression of *C4H* and *C3H* in lettuce, possibly explaining the enhanced CGA accumulation under continuous blue light and a high $CO_2$ concentration (Fig. 5). Under the same conditions, the expression of genes related to flavonoid biosynthesis was increased. Furthermore, a low $CO_2$ concentration may have promoted rutin accumulation by downregulating *DFR* expression under continuous blue light. In addition, a low air temperature, continuous blue light, and high $CO_2$ concentration further enhanced CGA accumulation by suppressing PPO activity in lettuce plants.

## 5. Conclusion

In edible fresh leafy vegetables such as lettuce, water-soluble polyphenols such as CGA are important nutrients. We report that light quality, photoperiod, PPFD, and $CO_2$ concentration modulated the CGA and rutin contents of lettuce, synergically. Furthermore, *PAL(2)*, *C4H* and *C3H* expressions and PAL activity in lettuce plants were affected by light quality, photoperiod, and $CO_2$ concentration, which led to an increase in CGA content. In addition, a low air temperature promoted CGA accumulation under continuous blue light and a high $CO_2$ concentration. Such metabolite

379 responses to multiple environmental factors are likely related to plant resilience to environmental
380 stress.

381

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388

389 **Figure captions**

390 Fig. 1. Wavelength distributions irradiated by different light sources used in this experiment. B and R
391 show the blue and red LEDs respectively.

392

393 Fig. 2. Chlorogenic acid (CGA) and Rutin contents per FW and DW in young leaf lettuce plants
394 grown under three different light qualities irradiated by different LEDs (B; blue LED, R; Red
395 LED),photoperiod (24h; continuous lighting, 12h; 12h day and 12h night), and $CO_2$ concentrations
396 (1,000 ppm and 400 ppm). Error bars show standard errors (n=3). The significance shows the effects
397 of each factor and cross effects by analysis of variance (A.V.) as * ($P < 0.05$), **($P < 0.01$) , ***($P <$

398 0.001) and NS (non-significant), and different letters within same figure show the significant
399 differences among treatments by Tukey's test ($P < 0.05$). During this experiment, plants were grown
400 at the PPFD level of 200 μmol $m^{-2}$ $s^{-1}$ by different blue spectrum LEDs. Atmospheric conditions
401 were controlled to keep constant levels, air temperature 25 °C and relative humidity 70%.
402
403 Fig. 3. Phenylalanine-ammonia-lyase (PAL) activities in young leaf lettuce plants grown under three
404 different light qualities irradiated by different LEDs (B; blue LED, R; Red LED),photoperiod (24h;
405 continuous lighting, 12h; 12h day and 12h night), and $CO_2$ concentrations (1,000 ppm and 400 ppm).
406 Error bars show standard errors (n=3). The significance shows the effects of each factor and cross
407 effects by analysis of variance (A.V.) as * ($P < 0.05$), **($P < 0.01$) , ***($P < 0.001$) and NS
408 (non-significant), and different letters within same column show the significant differences among
409 treatments by Tukey's test ($P < 0.05$). During this experiment, plants were grown at the PPFD level
410 of 200 μmol $m^{-2}$ $s^{-1}$ by different blue spectrum LEDs. Atmospheric conditions were controlled to
411 keep constant levels, air temperature 25 °C and relative humidity 70%.
412
413 Fig. 4. Chlorogenic acid (CGA) contents per FW and DW in young leaf lettuce plants grown under
414 three different light qualities irradiated by different LEDs (B; blue LED, R; Red LED) with the
415 photoperiod (24h; continuous lighting), and $CO_2$ concentrations (1,000 ppm). Error bars show

416 standard errors (n=3). The significance shows the effects of each factor and cross effects by analysis
417 of variance (A.V.) as * ($P < 0.05$), **($P < 0.01$) , ***($P < 0.001$) and NS (non-significant), and
418 different letters within same figure show the significant differences among treatments by Tukey's test
419 ($P < 0.05$). During this experiment, plants were grown at the PPFD level of 200 μmol $m^{-2}$ $s^{-1}$ by
420 different blue spectrum LEDs. Atmospheric conditions were controlled to keep constant levels, air
421 temperature 15, 20 and 25 °C, respectively in each air temperature treatment (Air temp.) and relative
422 humidity 70%.
423
424 Fig. 5. A schematic model of a hypothesis for explaining the increases of chlorogenic acid and rutin
425 contents in lettuce plants grown under complex multi environmental factors. Abbreviations show, BL,
426 Blue light; CL, Continuous lighting; $HCO_2$, $CO_2$ concentration of 1,000 ppm; $LCO_2$, $CO_2$
427 concentration of 400ppm; LT, Low temperature; *PAL*, *phenylalanine ammonia-lyase*; *C4H*, *cinnamic*
428 *acid 4-hydroxylase*; *4CL*, *4-coumaroyl:CoA-ligase*; *HCT*, *hydroxycinnamoylcoenzyme A shikimate*
429 *hydroxycinnamoyl transferase*; *C3H*, *p*-coumarate *3-hydroxylase*; *HQT*, *hydroxycinnamoylcoenzyme*
430 *A quinate hydroxycinnamoyl transferase*; *CHS*, *chalcone synthase*; *CHI*, *chalcone isomerase*; *F3H*,
431 *flavanone 3-hydroxylase*; *FLS*, *flavonol synthase*; *DFR*, *dihydroflavonol 4-reductase*; *ANS*,
432 *anthocyanidin synthase*, GlcT: *glucosyl transferase*, RhaT: *rhamnosyl transferase*, PPO: polyphenol
433 oxidase, respectively.

434

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590 Tables

591 Table 1 Results of transcriptomic analysis related into the biosynthesis of
592 phenylpropanoids and flavonoids in lettuce plants grown under
593 blue or red LED light condition with continuous lighting and 1,000
594 ppm of $CO_2$ concentration.

| Genes[y] | RPKM R[z] | RPKM B | $log_2$(fc)(B/R) |
|---|---|---|---|
| *PAL(1)* | 16.7 | 63.4 | 1.92 |
| *PAL(2)* | 108.8 | 459.0 | 2.08* |
| *C4H* | 58.8 | 213.8 | 1.86 |
| *4CL* | 54.7 | 79.6 | 0.54 |
| *CHS(1)* | 474.1 | 1270.7 | 1.42 |
| *CHS(2)* | 110.2 | 1525.8 | 3.79* |
| *CHI(1)* | 2.8 | 5.2 | 0.91 |
| *CHI(2)* | 67.8 | 826.2 | 3.61* |
| *CHI(3)* | 77.9 | 307.1 | 1.98 |
| *F3H* | 135.4 | 602.9 | 2.15* |
| *DFR* | 264.7 | 748.1 | 1.50 |
| *ANS* | 78.6 | 158.6 | 1.01 |
| *C3H* | 153.1 | 244.2 | 0.67 |
| *FLS* | 105.4 | 831.6 | 2.98* |
| *UGT78D2* | 194.0 | 666.2 | 1.78 |
| *MYB90/PAP2(1)* | 6.3 | 3.9 | -0.70 |
| *MYB90/PAP2(2)* | 52.1 | 36.6 | -0.51 |
| *KNOX* | 2.4 | 0.7 | -1.80 |

595 z: R and B mean the light quality conditions irradiated by red and
596 blue LED treatments, respectively
597 y: * shows that there are significant differences between light quality
598 treatments at $p$=0.05 level and |log2(fc)| > 2 in those genes.
599 z: Abbreviations of genes, *PAL*, *phenylalanine ammonia-lyase*; *C4H*,
600 *cinnamic acid 4-hydroxylase*; *4CL*, *4-coumaroyl CoA-ligase*; *C3H*,
601 *p-coumarate 3-hydroxylase*; *CHS*, *chalcone synthase*; *CHI*, *chalcone*
602 *isomerase*; *F3H*, *flavanone 3-hydroxylase*; *FLS*, *flavonol synthase*; *DFR*,
603 *dihydroflavonol 4-reductase*; *ANS*, *anthocyanidin synthase*.

604

605

606 Table 2 Effects of light qualities, photoperiods and $CO_2$ concentrations on the gene expressions on polyphenols and flavonoids synthesis
607 in young leaf lettuce plants grown for two days.

| Factors | Environmental conditions[z] | | | | | | | | Analysis of variance | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.Light quality | Blue | | | | Red | | | | | | | | | |
| 2.Photoperiod | 24h | | 12h | | 24h | | 12h | | | | | | | |
| 3.$CO_2$ | 1000 | 400 | 1000 | 400 | 1000 | 400 | 1000 | 400 | 1. | 2. | 3. | 1.× 2. | 1. × 3. | 2. × 3. |
| *PAL(1)*[x] | 0.037$^{-}$ | 0.032$^{-}$ | 0.025$^{-}$ | 0.051$^{-}$ | 0.017$^{-}$ | 0.014$^{-}$ | 0.028$^{-}$ | 0.009$^{-}$ | * | - | - | - | - | - |
| *PAL(2)* | 0.674$^{a}$ | 0.469$^{ab}$ | 0.361$^{b}$ | 0.281$^{bc}$ | 0.123$^{c}$ | 0.069$^{c}$ | 0.100$^{c}$ | 0.045$^{c}$ | *** | ** | * | * | - | - |
| *C4H* | 0.621$^{a}$ | 0.310$^{b}$ | 0.699$^{a}$ | 0.164$^{b}$ | 0.079$^{b}$ | 0.135$^{b}$ | 0.189$^{b}$ | 0.144$^{b}$ | *** | - | *** | - | * | *** |
| *4CL* | 0.265$^{-}$ | 0.139$^{-}$ | 0.219$^{-}$ | 0.169$^{-}$ | 0.048$^{-}$ | 0.192$^{-}$ | 0.187$^{-}$ | 0.195$^{-}$ | - | - | - | - | - | * |
| *C3H* | 0.111$^{-}$ | 0.086$^{-}$ | 0.270$^{-}$ | 0.167$^{-}$ | 0.450$^{-}$ | 0.057$^{-}$ | 0.124$^{-}$ | 0.094$^{-}$ | - | - | * | * | - | - |
| *CHS* | 1.398$^{a}$ | 0.858$^{ab}$ | 0.425$^{ab}$ | 0.278$^{ab}$ | 0.028$^{b}$ | 0.052$^{b}$ | 0.044$^{b}$ | 0.027$^{b}$ | *** | * | - | * | - | - |
| *CHI(2)* | 0.642$^{-}$ | 0.475$^{-}$ | 0.483$^{-}$ | 0.362$^{-}$ | 0.023$^{-}$ | 0.039$^{-}$ | 0.028$^{-}$ | 0.020$^{-}$ | *** | - | - | - | - | - |
| *F3H* | 0.187$^{-}$ | 0.247$^{-}$ | 0.290$^{-}$ | 0.247$^{-}$ | 0.026$^{-}$ | 0.054$^{-}$ | 0.081$^{-}$ | 0.034$^{-}$ | ** | - | - | - | - | - |
| *DFR* | 0.025$^{-}$ | 0.008$^{-}$ | 0.019$^{-}$ | 0.003$^{-}$ | 0.017$^{-}$ | 0.007$^{-}$ | 0.011$^{-}$ | 0.001$^{-}$ | - | - | ** | - | - | - |
| *ANS* | 0.003$^{-}$ | 0.003$^{-}$ | 0.006$^{-}$ | 0.017$^{-}$ | 0.002$^{-}$ | 0.003$^{-}$ | 0.003$^{-}$ | 0.001$^{-}$ | - | - | - | - | - | - |
| *FLS* | 0.929$^{-}$ | 1.087$^{-}$ | 1.186$^{-}$ | 0.948$^{-}$ | 0.069$^{-}$ | 0.146$^{-}$ | 0.076$^{-}$ | 0.083$^{-}$ | ** | - | - | - | - | - |

608
609 z:Atmospheric conditions were controlled to keep constant levels, air temperature 25°C and relative humidity 70%. The PPFD was set as
610 200 μmol $m^{-2}$ $s^{-1}$ irradiated by blue or red LEDs.
611 y: The gene expression was normalized to the expression of the *ACTIN* gene. Each value shows the averaged mean ($n$ = 3). The significance
612 shows the effects of each factor and cross effects by analysis of variance (A.V.) as * ($P < 0.05$), **($P < 0.01$) , ***($P < 0.001$) and -
613 (non-significant), and different letters within same column show the significant differences among treatments by Tukey's test ($P < 0.05$).
614 x: See table 1.
615

Table 3 Effects of air temperatures on the gene expressions on polyphenols and flavonoids synthesis in young leaf lettuce plants grown for two days

| Genes[x] | Air temperatures | | |
|---|---|---|---|
| | 25°C | 20°C | 15°C |
| *PAL(1)* | 0.15[a] | 0.09[a] | 0.17[a] |
| *PAL(2)* | 2.89[a] | 2.49[a] | 2.85[a] |
| *C4H* | 2.42[a] | 0.72[b] | 1.04[b] |
| *4CL* | 0.55[a] | 0.41[a] | 0.35[a] |
| *C3H* | 0.45[a] | 0.31[a] | 0.64[a] |
| *CHS* | 1.15[a] | 5.22[ab] | 7.87[b] |
| *CHI(2)* | 0.87[a] | 1.74[a] | 5.78[b] |
| *F3H* | 0.46[a] | 0.76[a] | 2.52[b] |
| *DFR* | 1.91[a] | 2.19[a] | 6.25[a] |
| *ANS* | 0.24[a] | 2.67[a] | 0.74[a] |
| *FLS* | 1.02[a] | 1.58[a] | 0.11[a] |

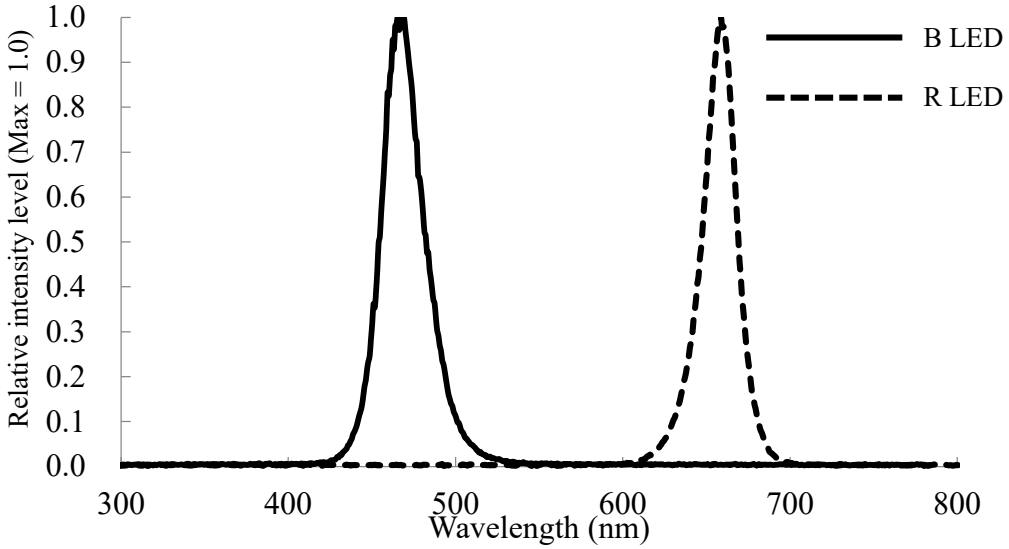
z:Atmospheric conditions were controlled to keep constant levels, each air temperature series and relative humidity 70%. The PPFD was set as 200 μmol $m^{-2}$ $s^{-1}$ (PPFD) irradiated by blue LED, continuously. $CO_2$ concentration was kept at 1,000 ppm level.

y:Each value shows the averaged mean ($n$ = 3). The different letters within same column show the significant differences among treatments by Tukey's test ($P < 0.05$).

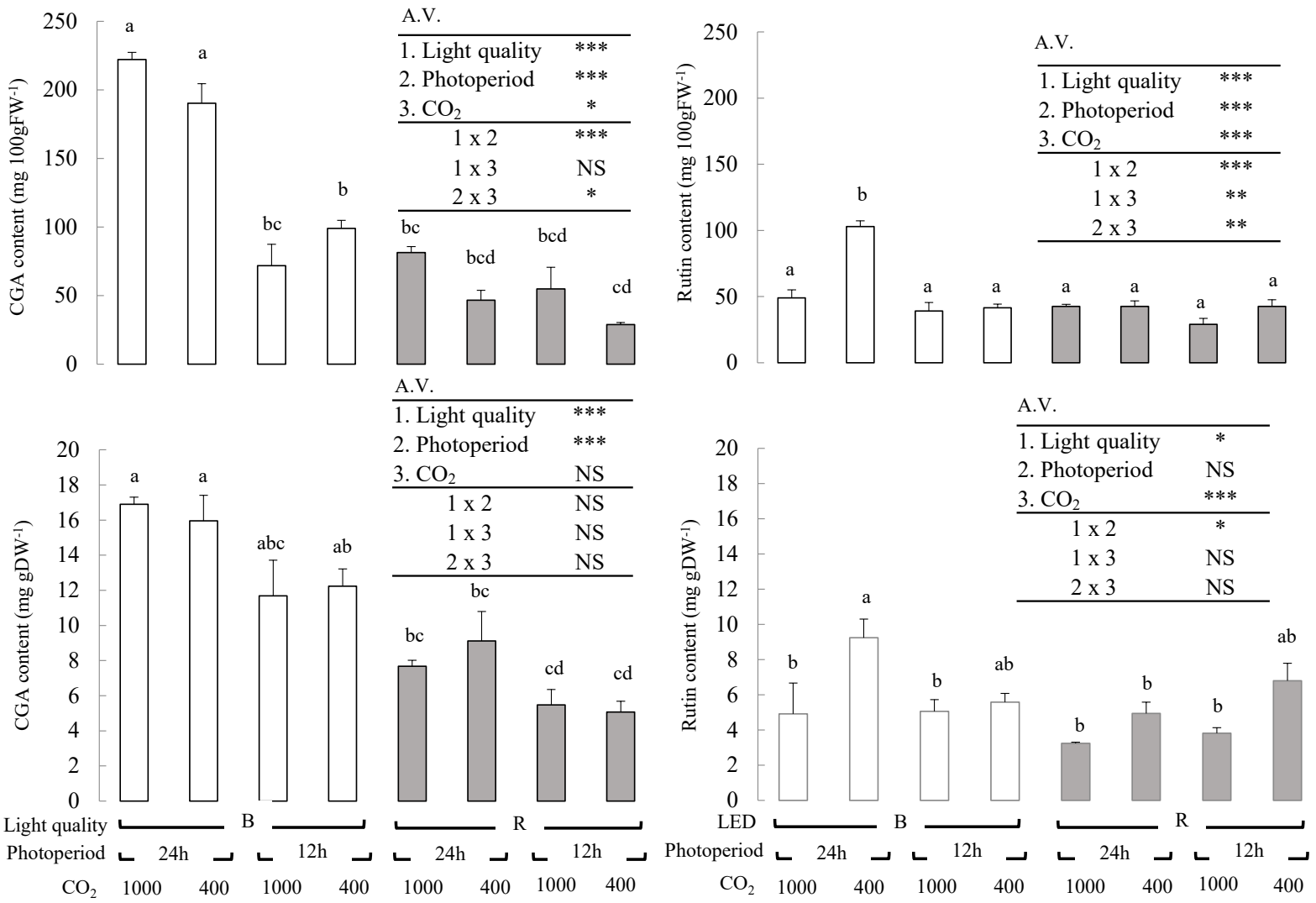
x: See table 1.

Figures

Fig. 1.

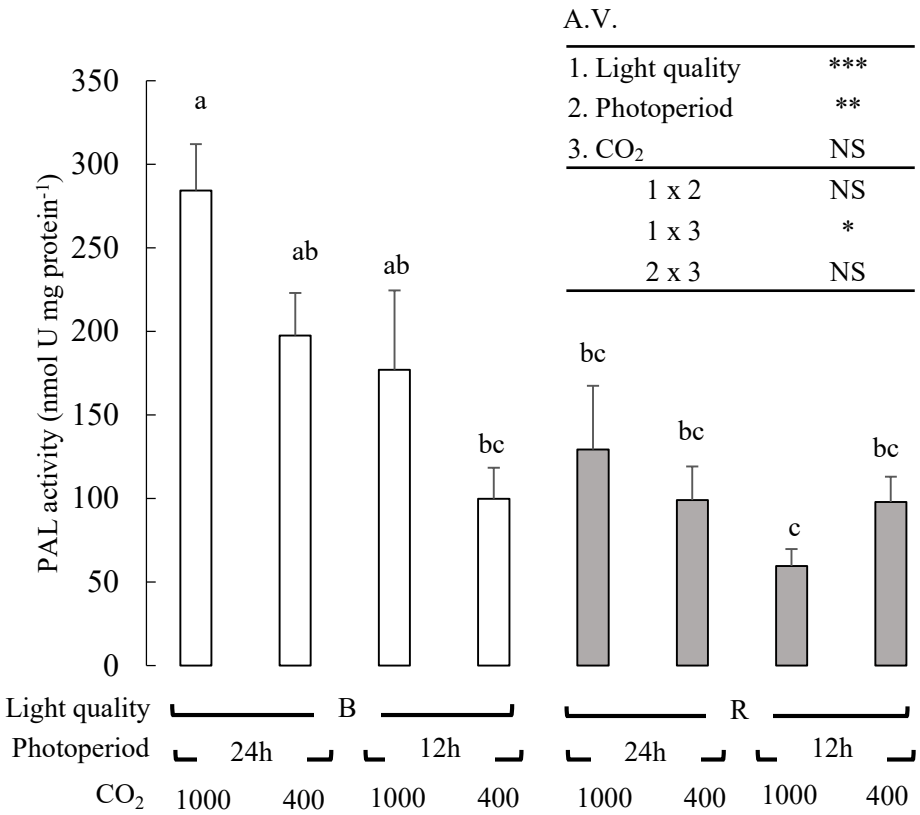


649 Fig. 2.


CGA content (mg 100gFW-1)
250
200
150
100
50
0
a
a
bc
b
bc
bcd
bcd
cd
A.V.
1. Light quality ***
2. Photoperiod ***
3. CO2 *
1 x 2 ***
1 x 3 NS
2 x 3 *
Rutin content (mg 100gFW-1)
a
b
a
a
a
a
a
a
A.V.
1. Light quality ***
2. Photoperiod ***
3. CO2 ***
1 x 2 ***
1 x 3 **
2 x 3 **
CGA content (mg gDW-1)
20
18
16
14
12
10
8
6
4
2
0
a
a
abc
ab
bc
bc
cd
cd
A.V.
1. Light quality ***
2. Photoperiod ***
3. CO2 NS
1 x 2 NS
1 x 3 NS
2 x 3 NS
Rutin content (mg gDW-1)
b
a
b
ab
b
b
b
ab
A.V.
1. Light quality *
2. Photoperiod NS
3. CO2 ***
1 x 2 *
1 x 3 NS
2 x 3 NS
Light quality B R
LED B R
Photoperiod 24h 12h 24h 12h
CO2 1000 400 1000 400 1000 400 1000 400

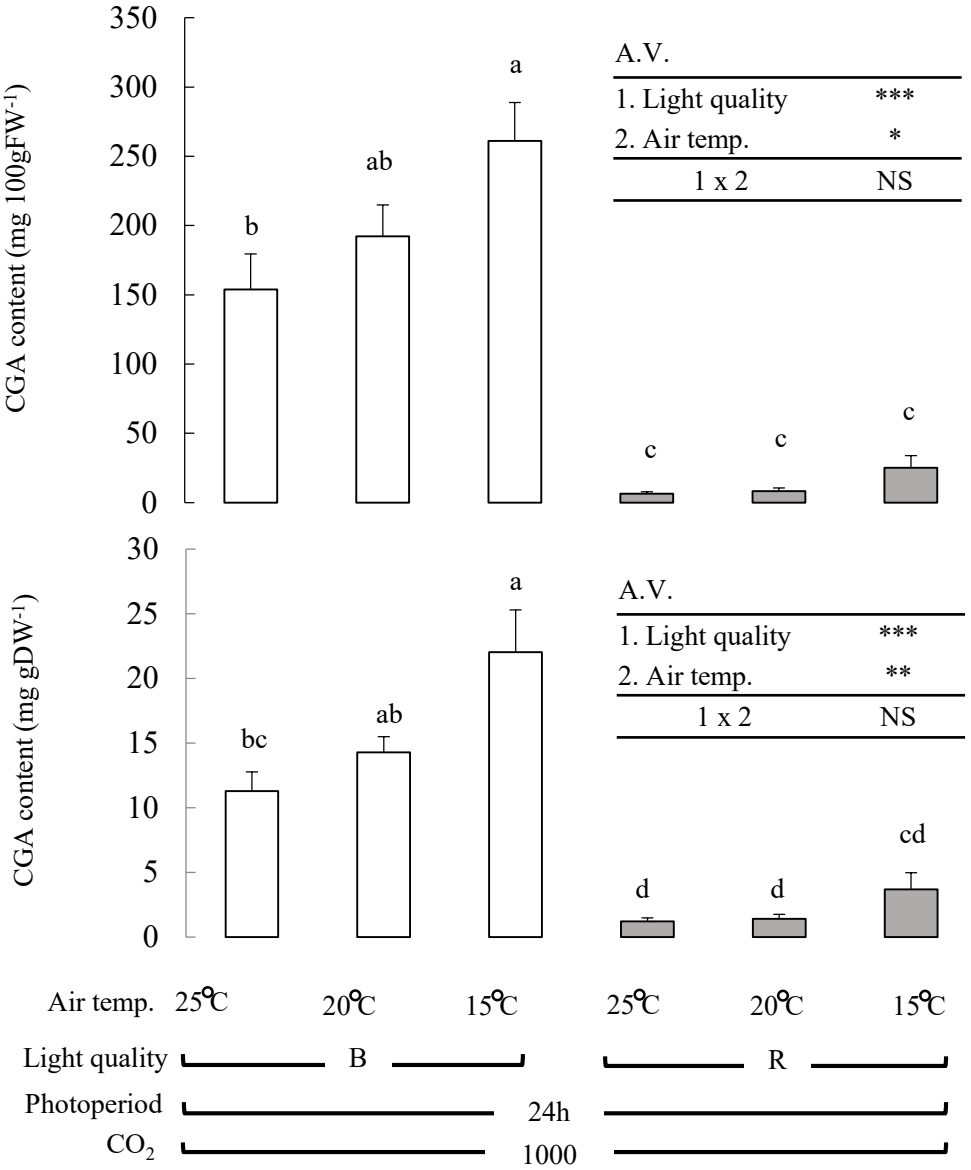
650

651 Fig. 3.
652

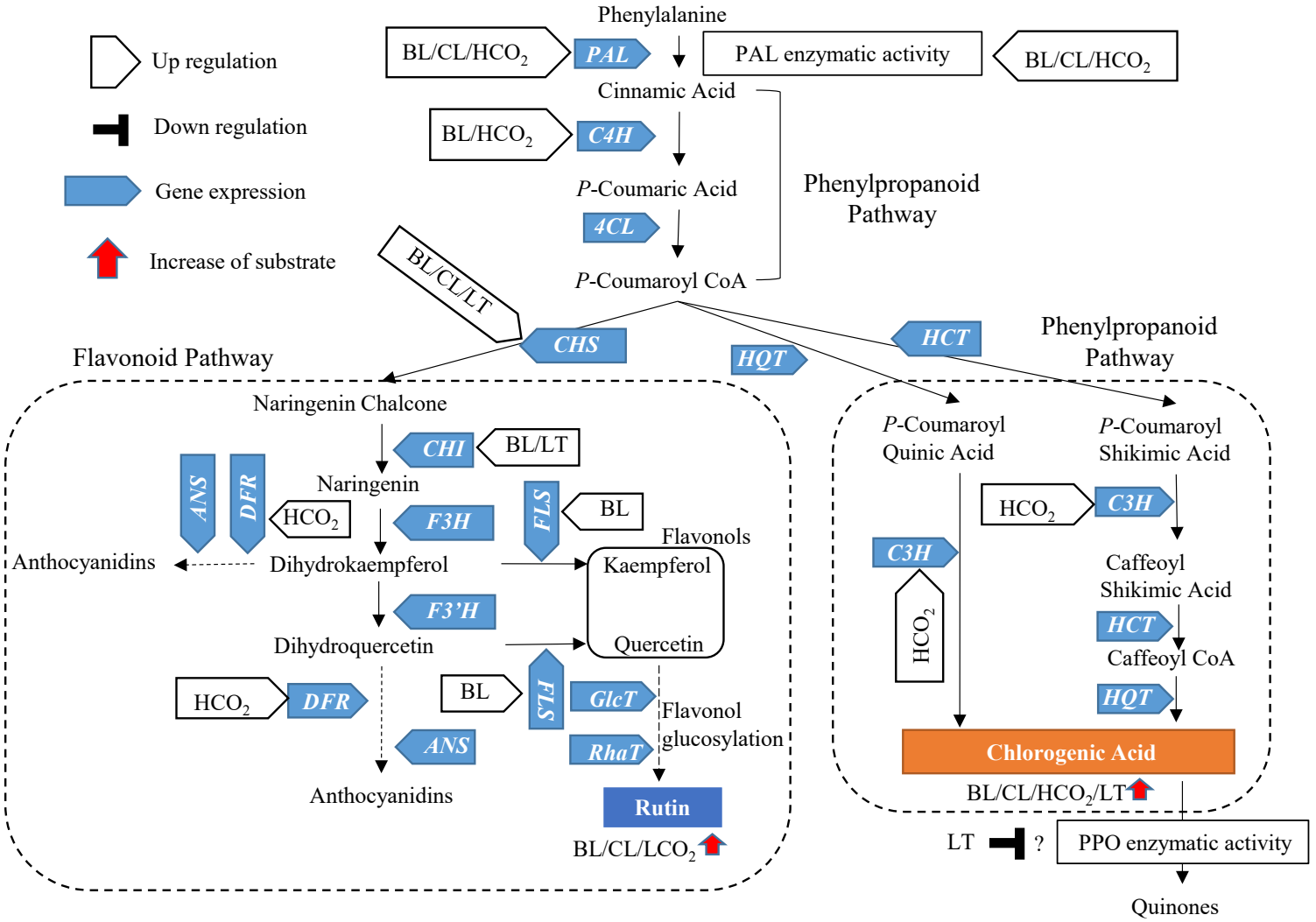



653
654
655

Fig. 4


CGA content (mg 100gFW-1)
350
300
250
200
150
100
50
0
b
ab
a
c
c
c
A.V.
1. Light quality ***
2. Air temp. *
1 x 2 NS
CGA content (mg gDW-1)
30
25
20
15
10
5
0
bc
ab
a
d
d
cd
A.V.
1. Light quality ***
2. Air temp. **
1 x 2 NS
Air temp. 25℃ 20℃ 15℃ 25℃ 20℃ 15℃
Light quality B R
Photoperiod 24h
CO2 1000

660 Fig. 5.


Up regulation
Down regulation
Gene expression
Increase of substrate
Phenylalanine
BL/CL/HCO2
PAL
PAL enzymatic activity
BL/CL/HCO2
Cinnamic Acid
BL/HCO2
C4H
P-Coumaric Acid
4CL
P-Coumaroyl CoA
Phenylpropanoid Pathway
BL/CL/LT
CHS
HQT
HCT
Phenylpropanoid Pathway
Flavonoid Pathway
Naringenin Chalcone
CHI
BL/LT
ANS
DFR
Naringenin
HCO2
F3H
FLS
BL
Flavonols
Anthocyanidins
Dihydrokaempferol
Kaempferol
F3'H
Dihydroquercetin
Quercetin
HCO2
DFR
BL
FLS
GlcT
Flavonol glucosylation
ANS
RhaT
Anthocyanidins
Rutin
BL/CL/LCO2
P-Coumaroyl Quinic Acid
P-Coumaroyl Shikimic Acid
HCO2
C3H
C3H
HCO2
Caffeoyl Shikimic Acid
HCT
Caffeoyl CoA
HQT
Chlorogenic Acid
BL/CL/HCO2/LT
LT
?
PPO enzymatic activity
Quinones

661