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Full Length Article

Ethylene is involved in Red Light-Induced Anthocyanin Biosynthesis in Cabbage (*Brassica oleracea*)

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Abstract

In plants, both light environments and phytohormones affect the biosynthesis of flavonoids. However, the role of ethylene in light quality-regulated changes in anthocyanin biosynthesis remains largely unclear. Here, two cultivars of cabbage were exposed to red, blue and white light for 10 days. Results showed that red light exposure for 10 d increased anthocyanin concentration by 15.34% and 18.29% compared with that under white light. Notably, exogenous ethylene treatment suppressed the anthocyanin concentration dose-dependently, showing the greater inhibition with the higher concentration of ethylene. Time course analysis of the activity of anthocyanin biosynthetic enzymes, such as phenylalanine ammonialyase (PAL), chalcone isomerase (CHI), dihydroflavonol 4-reductase (DFR) and UDP-glucose flavonoid 3-o-glucosyl transferase (UFGT), revealed that exogenous ethylene attenuated the red light-induced elevation in their activity, leading to decreased anthocyanin contents. More precisely, exogenous ethylene inhibited the activity of PAL, CHI, DFR and UFGT by 51.74%, 83.49%, 52.86% and 70.67%, respectively compared with the red light treatment on 10 d. Regression analysis showed that the red light or ethylene treatment affected the relationship between the anthocyanin content and PAL or DFR activity to some extent, however, the relationship between anthocyanin content and CHI or UFGT activity was relatively stable, suggesting that the relationship between anthocyanin content and some key enzymes responsible for its synthesis is largely dependent on both endogenous signals (ethylene) and environmental stimuli (red light) in cabbage. Our results suggest that ethylene acts as a negative regulator in red light-regulated anthocyanin biosynthesis in cabbage and thus may have potential implication in agronomic management of vegetables.

Keywords: Flavonoids; Light quality; Phenylalanine ammonia-lyase; Phytohormone; Secondary metabolism

Introduction

Anthocyanins are a kind of water-soluble natural pigments ubiquitous across the plant kingdom. They belong to the flavonoid class of secondary metabolites that largely determine the bright color (mostly purple) of leaves, stems, flowers, and fruits. Anthocyanins have numerous health benefits due to theantioxidant functions (Wang *et al*., 2018). Anthocyanins are synthesized *via* the phenylpropanoid pathway in plants, in which phenylalanine acts as the primary precursor and is catalyzed by a series of enzymes (Fig. 1), such as phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavnol-4-reductase(DFR), anthocyanidin synthase (ANS) and flavonoid 3-o-glucosyltransferase (UFGT) to give rise of anthocyanins as the end products (Hou *et al.,* 2017). All of these enzymes were encoded by their structural genes. The expression of these structural genes is dependent on the regulatory genes. Both structural genes and regulatory genes jointly constitute the molecular regulatory network of anthocyanin synthesis pathway in plants. Nonetheless, different environmental factors influence anthocyanin synthesis by inducing the expression of relevant genes involved in the biosynthesis of anthocyanins (David, 2000).

In addition to the environmental factors such as light, temperature and ozone, endogenous factors including sugar and phytohormones differentially affect the metabolism of anthocyanins (Abdullah *et al.,* 2018). In particular, the effect of light on anthocyanin synthesis has emerged as a research focus in recent years. The fact that the key enzymes responsible for the biosynthesis of anthocyanins, such as PAL, CHS, DFR and UFGT are largely regulated by light, changes in light conditions eventually affect anthocyanin accumulation in plants (Feng *et al.,* 2013; Lu *et al*., 2015). Nonetheless, the response greatly varies depending on the type of light and the species of plants (Jiang *et al*., 2016). In turnip, ultraviolet (UV)-A induces anthocyanin accumulation, however, the pattern of UV-A-induced anthocyanin synthesis is different from that induced by UV-B and blue light. Moreover, the sites of action of different light in the hypocotyl of turnip seedlings are different.

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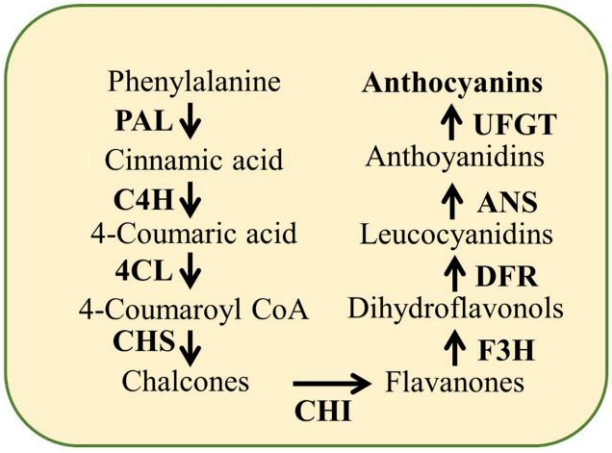

Fig. 1: Anthocyanin biosynthesis pathway in plants. Key enzymes such as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), p-coumarate:coa ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP- glucose flavonoid 3-o-glucosyl transferase (UFGT) are shown in bold. Adopted and redrawn from Li *et al.* (2017)

The blue light mainly impacts the upper part of the hypocotyl and the UV-B affects the middle and upper parts, while the UV-A induces anthocyanin in the lower part to the upper part of the hypocotyls (Wang *et al*., 2012).

Notably, blue light-induced anthocyanin synthesis is associated with the accumulation of abscisic acid (ABA) in grape skin (Kondo *et al*., 2014). In addition to ABA, phytohormones such as auxin, cytokinin and jasmonic acid play an important role in anthocyanin synthesis. For instance, exogenous indole acetic acid (IAA, a bioactive auxin) treatment upregulates the expression of *CHS, CHI, F3H, TTG1, PAP1* and *MYB12* in *Arabidopsis* seedlings (Lewis *et al*., 2011). Likewise, ABA promotes anthocyanin synthesis in grape (Zhu *et al*., 2016; Renata *et al*., 2018). Recent studies also revealed a role for melatonin in enhancing anthocyanin content in cabbage by up-regulating the expression of transcription factors, such as *MYB, bHLH,* and *WD40* (Zhang *et al*., 2016). *Arabidopsis* mutant *ga1* which is GA_3 deficient as well as insensitive shows an upregulated expression of *PAP1* gene alongside increased anthocyanin content (Loreti *et al*., 2008). When a mutated melon ethylene receptor gene (Cm-ETR1/H69A) is expressed in tobacco, anthocyanin accumulation elevates in petals (Takada *et al*., 2005). While some studies indicate the importance of ethylene in anthocyanin metabolism (Das *et al*., 2012; Gao *et al*., 2015; Barba-Espín *et al*., 2017; Zheng *et al*., 2018), the involvement of ethylene in light quality-regulated changes in anthocyanin biosynthesis remains far from being fully substantiated.

Given the key function of anthocyanins in imparting the specific color to petals, fruits, and seed skins, a good number of studies were focused on the regulation of anthocyanin content in plants (Katayama-Ikegami *et al*., 2016; Olivares *et al*., 2017). However, in some cases, higher anthocyanin concentration may not necessarily be beneficial to improving overall quality of crops. For example, in the spring 2018, the production of vegetables such as cabbage and lettuce was severely affected in the Zhangbei county of north China (East longitude 114°10′–115°27′, North latitude 40°57′–41°34′, 1600–1800 m above sea level) due to increased accumulation of anthocyanin, which turned the crops red (information provided by the local agriculture department). It was anticipated that changes in light quality potentially modulated plant metabolism towards reddening. Therefore, it is also important to explore the negative regulators of anthocyanin biosynthesis, which might help to overcome the aforementioned problems.

In the current study, we hypothesized that exposure of plants to red or blue light quality potentially enhanced anthocyanin biosynthesis which altered normal pigmentation in leaves. To test this hypothesis, we exposed two cultivars of cabbage to different light quality (red, blue and white) and measured anthocyanin concentration. In addition, we used exogenous ethylene to explore its role in red light-induced anthocyanin biosynthesis in cabbage. The study advances our current understanding of the light quality-regulated changes in plant secondary metabolism and provides useful references for agronomic management of vegetables.

Materials and Methods

Plant Materials

Seeds of two cabbage (*Brassica oleracea* L.) cultivars namely, Japan Outstanding and Zhonggan 21 (both genotypes show characteristics green leaf phenotype) were sown in pots (12 cm × 10 cm) filled with medium, a mixture of peat soil and vermiculite (3:1 w/w), and then covered with medium about 0.5–1 cm. Seedlings were cultured carefully until they were used for further study at three-leaf stage.

Screening for Variety and Light Quality

Uniform size healthy seedlings at three-leaf stage were selected and grown in controlled environment chambers under different light quality supplied with light-emitting photodiodes (LED, made by Guangdong Chenhua Co., Ltd., Shenzhen, China), such as white light (control), red light, and blue light with light intensity 80 μmol $m^{-2}.s^{-1}$, temperature 20/15℃ (day/night). Leaf samples were harvested every two days (at 10.00 a.m.) after the treatments up to 10 days to determine the content of anthocyanins. Morphological parameters were measured after 10 days of light treatment.

Ethylene Treatment

Selected healthy and uniform size seedlings of Zhonggan 21 at three-leaf stage were grown under red light (light intensity 80 μmol $m^{-2}.s^{-1}$, temperature 20/15℃) and sprayed with different concentrations of 2-chloroethylphosphonic acid (ethephon, Shanghai Puzhen biotechnology co, Ltd, Shanghai China), which is readily absorbed by the plants and releases ethylene. The treatments were 0 mg L^{-1} 2-chloroethylphosphonic acid + 0.1% Tween 80, 20 mg L^{-1} 2-chloroethylphosphonic acid + 0.1% Tween 80, 40 mg L^{-1} 2-chloroethylphosphonic acid + 0.1% Tween 80, 60 mg L^{-1} 2-chloroethylphosphonic acid + 0.1% Tween 80 and 80 mg L^{-1} 2-chloroethylphosphonic acid + 0.1% Tween 80. For simplicity, we referred the treatments 0 mg L^{-1} ethylene (control), 20 mg. L^{-1} ethylene, 40 mg L^{-1} ethylene, 60 mg L^{-1} ethylene and 80 mg L^{-1} ethylene, respectively. Seedlings were sprayed with ethephon 3 times a day with a 30 min interval for the first 3 days during light hours. Leaf samples were harvested every two days (at 10.00 a.m.) after the treatments up to 10 days to assay the content of anthocyanins in order to choose the best concentration of ethylene that could effectively inhibit anthocyanin accumulation. Thus, the most effective concentration (80 mg L^{-1}) of ethylene was chosen for the following experiment.

Selected healthy and uniform size seedlings of Zhonggan 21 at three-leaf stage were grown under red light and white light (light intensity 80 μmol $m^{-2}.s^{-1}$, temperature 20/15℃). Seedlings under red light treatment were sprayed with 80 mg L^{-1} 2-chloroethylphosphonic acid, 3 times a day with a 30 min interval for the first 3 days. Leaf samples were harvested every two days (at 10.00 a.m.) after the treatments to determine the activity of the biosynthetic enzymes.

Anthocyanin Content Determination

Anthocyanin content was assayed according to the method of Su *et al*. (2017). Leaf samples (1 g) were incubated overnight in 10 mL of 1% (v/v) HCl in methanol at room temperature. OD of the extracts was measured at 530 and 600 nm spectrophotometrically (A530–A600) (UV-5200 spectrophotometer, Shanghai Metash Instruments Co. Ltd, Shanghai, China). Anthocyanin content was finally presented on a fresh weight basis.

Enzymes Extraction and Activity Measurement

Enzymes were extracted by the method of Niu *et al*. (2017) with some modification. 1g of leaves was ground in 5 mL 100 mM Tris-HCl (pH7.0) containing 14 mM β-mercaptoethanol, 5 mM DDT, 1% BAS and 5% PVPP. After centrifugation at 12,000 rpm at 4℃ for 30 min, the supernatants were used for the analysis of enzyme activity.

PAL activity was assayed according to the method of Su *et al*. (2017). The enzyme extracts (approximately 2.75 mL) were incubated with a medium containing 1.75 mL of boric acid-borax buffer (pH 8.8), 0.5 mL crude enzyme and 0.5 mL of 10 mg/mL L-phenylalanine as substrate at 35℃ for 30 min. After centrifugation at 5000 rpm at 4℃ for 5 min, the reaction was terminated by adding 1 mL of 15% HCl. PAL activity was measured by the change in absorbance at 290 nm. One unit was defined as a 0.01 change in absorbance at 290 nm every min. PAL activity was finally presented on a protein basis.

CHI activity was assayed according to the method of Huang *et al*. (2011). 0.8 mL of enzyme extracts was added to 2 mL 50 m*M* Tris-HC1 (pH7.4，containing 7.5 m*M* BSA, 50 m*M* KCN), then added 50 μg mL^{-1} chalcone and incubated at 34℃ for 30 min. The changes in absorbance at 381 nm were measured to determine CHI activity.

DFR activity was assayed according to the method of Niu *et al*. (2017). The incubation mixture contained 300 μM dihydroquercetin, 20 μM NADPH, 1 m*M* DDT, 0.4 mL of enzyme extract, and a system consisting of 5 units of glucose-6-phosphate dehydrogease and 0.1 m*M* of glucose-6-phosphate. The assay mixture was incubated at 35℃ for 1 h and then extracted twice with 1.0 mL ethyl acetate. The ethyl acetate fraction was evaporated to dryness under a stream of nitrogen gas. The numbers of molecules of leucocyanidin in the ethyl acetate fraction were estimated based on the acid-catalyzed conversion of leucocyanidin to cayanidin, which was accomplished by the addition of 1 mL of butanol-HCl (95:5, v/v) reagent to the evaporated ethyl acetate fraction and heated at 95℃ for 15 min. The absorbance at 550 nm was monitored. The extinction coefficient was 34.7 $mM^{-1}.cm^{-1}$. Control assays were conducted using complete assays with heated inactivated enzyme.

UFGR activity was assayed according to the method of Huang *et al*. (2011). The reaction mixture contained 100 μL 50 m*M* Tris-gly (pH 8.5), 10 μL 15 m*M* UDP-glucose, 15 μL 2 m*M* quercetin and 200 μL of enzyme extracts. After incubation for 30 min at 30℃, the reaction was terminated by addition of 75 μL 20% TCA. UFGT activity was measured by the change in absorbance at 351 nm. One unit was defined as a 0.01 change in absorbance at 351 nm every min.

Statistical Analysis

Data were subject to analysis of variance (ANOVA) and analyzed using SPSS19.0 statistical software package. Significant differences between the means were separated by Duncan' s multiple range test ($P \leqq 0.05$).

Results

Effects of Different Light on the Content of Anthocyanin and Plant Growth

To assess the effects of light quality on anthocyanin content,

we exposed two genotypes of cabbage, namely Japan outstanding and Zhonggan 21 to three kinds of light, such as blue, red and white light. Results showed that exposure of plants to red light gradually increased the levels of anthocyanin in both genotypes (Fig. 2). More precisely, anthocyanin content in Zhonggan 21 increased by 13.74% on the 8th day and 15.34% on the 10th day compared with control. While in Japan outstanding, it increased by 19.05% and 18.29%, respectively. However, the blue light caused no significant effect on anthocyanin content in Zhonggan 21 during the whole period of treatment. Where as in Japan outstanding, the anthocyanin content increased by 8.67% compared with the control on the 10th day under blue light. Notably, the content of anthocyanins in Zhonggan 21 was consistently higher than that of Japan outstanding regardless of the light quality, so, we chose Zhonggan 21 and red light treatment for subsequent experiments.

We also measured different morphological parameters after exposure of two cabbage cultivars to different light quality for 10 days. As shown in Table 1, the plant height of Zhonggan 21 under red light was the highest among all treatments, while the plant height under blue light was the lowest in both cultivars. Meanwhile, fresh weight of both cultivars increased significantly under red and blue light compared with that under white light. Compared with the white light and red light, the stem diameter under blue light significantly increased only in Zhonggan 21 cultivar. However, there was no significant difference in leaf number under different light in each cultivar.

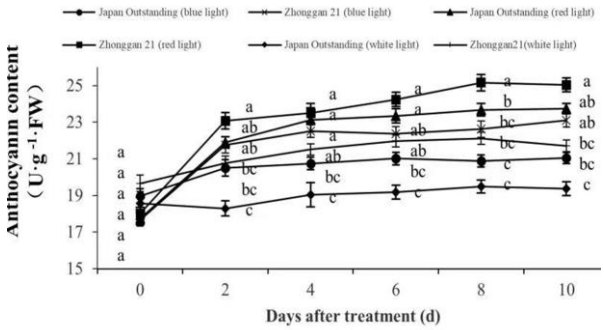

Fig. 2: Effects of light quality on anthocyanin content in leaves of two cabbage cultivars. At 3-leaf stage, two cabbage cultivars, Japan Outstanding and Zhonggan 21, were exposed to different LED light, such as white light (control), red light, and blue light with light intensity 80 μmol. m^{-2}. s^{-1}, photoperiods 12 h·d^{-1}. Leaf samples were harvested every two days after the treatments up to 10 days to determine the content of anthocyanins. Data are the mean ± SD of 3 replicates. Means followed by different letters at the same time point are significantly different at $P \leqq 0.05$ as determined by Duncan's multiple range tests

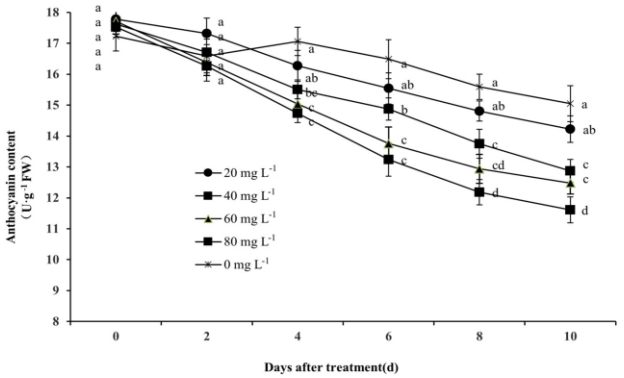

Fig. 3: Effects of different concentration of ethylene on anthocyanin content in leaves of cabbage. At 3-leaf stage, seedlings of cabbage cultivar Zhonggan 21, were exposed to red light and sprayed with different concentrations (0, 20, 40, 60 and 80 mg L^{-1}) of ethylene. Leaf samples were harvested every two days after the treatments up to 10 days to determine the content of anthocyanins. Data are the mean ± SD of 3 replicates. Means followed by different letters at the same time point are significantly different at $P \leqq 0.05$ as determined by Duncan's multiple range tests

Effects of Exogenous Ethylene on the Content of Anthocyanins

Next, we measured the content of anthocyanins with or without different concentrations of ethylene treatment under red light conditions. As shown in Fig. 3, ethylene treatment gradually and drastically decreased the content of anthocyanins in a dose-dependent manner particularly from the 4th day. The maximum attenuation was observed following the highest dose of ethylene (80 mg L^{-1}), which decreased anthocyanin content by 25.51% on the 10th day compared with the control (0 mg L^{-1}). On the same day, 20, 40 and 60 mg L^{-1} ethylene decreased the content of anthocyanins by 14.49, 17.08 and 22.86%, respectively. This implies that ethylene-induced suppression of anthocyanin content is dependent on both exogenous ethylene concentration and time progression following the treatment.

Impacts of Ethylene on the Activity of Anthocyanin Biosynthesis Enzymes

Changes of PAL Activity: As shown in Fig. 4, PAL activity was significantly different between red light and control group. PAL activity in the control group did not change over time after treatment, while PAL activity under red light initially increased sharply up to 4 d, reaching the peak, and then declined rapidly until 6 d post treatment. No remarkable changes were noticed in the anthocyanin contents from 6 d to 10 d post treatment. PAL activity under red light was significantly higher than that of the control group at the first four days. However, exogenous ethylene treatment significantly attenuated the effect of red light on PAL activity during the whole period of treatment. At the end of the experiment, the PAL activity decreased by 57.14% in the red light + ethylene-treated plants compared with that of the only red light-treated plants.

Table 1: Different morphological parameters as influenced by the exposure of plants to different light quality for 10 days in two cabbage cultivars

Cabbage cultivars	Light treatment	Fresh weight (g plant^{-1})	Stem diameter (cm)	Plant height (cm)	Leaf number (plant^{-1})
Zhonggan 21	red light	4.97 ± 014a	0.39 ± 0.04b	4.98 ± 0.17a	4.51 ± 0.01ab
	blue light	4.91 ± 0.12a	0.48 ± 0.02a	4.00 ± 0.14d	4.88 ± 0.02a
	white light	3.72 ± 0.04b	0.38 ± 0.05b	4.44 ± 0.10bc	4.61 ± 0.03a
Japan Outstanding	red light	4.84 ± 0.08a	0.32 ± 0.04b	4.37 ± 0.46c	4.31 ± 0.02b
	blue light	4.79 ± 0.06a	0.33 ± 0.02b	4.06 ± 0.06d	4.58 ± 0.04ab
	white light	3.72 ± 0.11b	0.35 ± 0.05b	4.55 ± 0.41b	4.25 ± 0.05b

Means ± SD with the different letters differ significantly at $P \leqq 0.05$, n= 10

Changes of CHI activity: As shown in Fig. 5, the activity of CHI of the control did not change over the time in the control group. While under the red light, CHI activity gradually increased particularly from 2 to 4 d post treatment, reaching the peak on the 4th day. Afterward, the CHI activity increased slightly. After 10 d of red light treatment, the CHI activity increased by 178.99% compared with that of control. However, ethylene treatment significantly inhibited the CHI activity by 83.49% compared with the red light treatment on the 10th d post treatment.

Changes of DFR activity: While DFR activity in the control group did not change with the progression of time, it rapidly increased until 4 d and then decreased sharply under the red light (Fig. 6). More specifically, DFR activity peaked at the 4 d post treatment, followed by a sudden decline, which became more or less stable from 6 to 10 d post treatment. However, exogenous ethylene treatment inhibited the red light-induced transient increase in DFR activity. The activity of DFR decreased by 52.86% compared with the red light treatment on the 10th d post treatment.

Changes of UFGT activity: As shown in fig. 7, UFGT activity showed a slow gradual increase under the red light. During the first 8 days, UFGT activity increased almost in a consistent magnitude reaching its peak at the 8th d post treatment, and then it declined slightly. In contrast, exogenous ethylene treatment significantly inhibited the effect of red light on the activity of UFGT. Since the 4th day, UFGT activity was significantly lower than that of red light group. With the extension of time, the activity decreased continuously under ethylene treatment, and it decreased by 70.67% on the 10th d post treatment compared with the only red light treatment.

These results revealed that red light increased the activity of enzymes involved in anthocyanin biosynthesis, such as PAL, CHI, DFR and UFGT, however, exogenous ethylene could significantly attenuate the red light-induced elevation in the activity of these enzymes.

Table 2: Regression model between anthocyanin content and activity of related enzymes

Treatment	Regression model	Correlation coefficient	F	x
Red light	y=-0.007x^2+1.15x-17.14	0.67*	0.92	PAL
	y=1.03x+17.61	0.79*	6.42	CHI
	y=0.0001x^2+0.09x+9.00	0.54	0.62	DFR
	y=10.72x+17.41	0.90*	6.41	UFGT
Red light+ethylene	y=0.21x+8.71	0.63*	2.62	PAL
	y=3.25x+9.65	0.99**	209.82	CHI
	y=-0.001x^2+0.42x-17.06	0.99**	106.34	DFR
	y=32.83x+6.72	0.99**	197.31	UFGT

** and * indicated significant at 0.01 and 0.05, respectively; y: anthocyanin content, x: activity of related enzyme, phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), dihydroflavonol 4-reductase (DFR), UDP- glucose flavonoid 3-o-glucosyl transferase (UFGT)

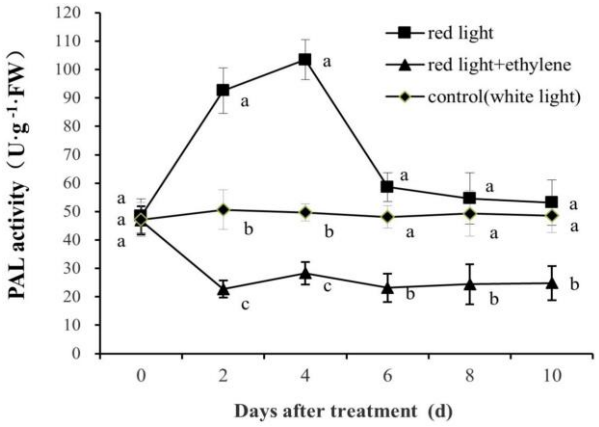

Fig. 4: Interactive effects of red light and exogenous ethylene on phenylalanine ammonia-lyase (PAL) activity in cabbage leaves. At 3-leaf stage, seedlings of cabbage cultivar Zhonggan 21 were exposed to red light and sprayed with 80 mg L^{-1} ethylene. Leaf samples were harvested every two days after the treatments up to 10 days to determine the enzyme activity. Data are the mean ± SD of 3 replicates. Means followed by different letters at the same time point are significantly different at $P \leqq 0.05$ as determined by Duncan's multiple range test

Regression Analysis between Anthocyanin Content and the Activity of Related Enzymes

Table. 2 showed the relationship between anthocyanin content and enzyme activity. Under red light, anthocyanin content and PAL activity showed a quadratic curve, but under red light + ethylene treatment, the relationship was linear. There was no significant relationship between anthocyanin content and DFR activity under red light, while under red light + ethylene treatment it was a significant quadratic relationship. Under the red light and red light + ethylene treatments, the relationships between anthocyanin content and the activity of CHI and UFGT were all linear. Thus it could be concluded that the relationship between

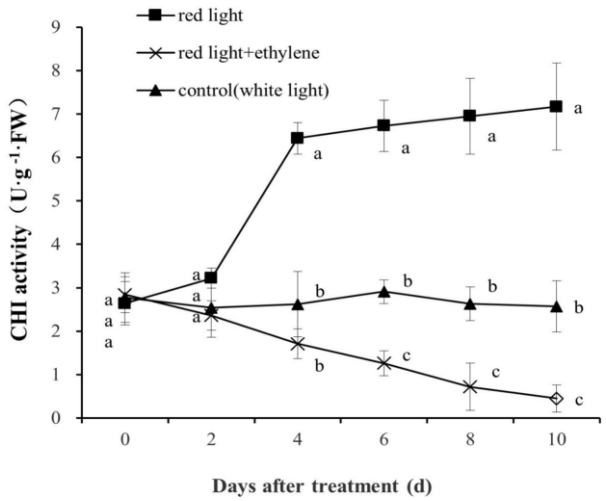

Fig. 5: Effect of exogenous ethylene on red light-induced chalcone isomerase (CHI) activity in cabbage leaves. Data are the mean ± SD of 3 replicates. Means followed by different letters at the same time point are significantly different at $P \leqq 0.05$ as determined by Duncan's multiple range test

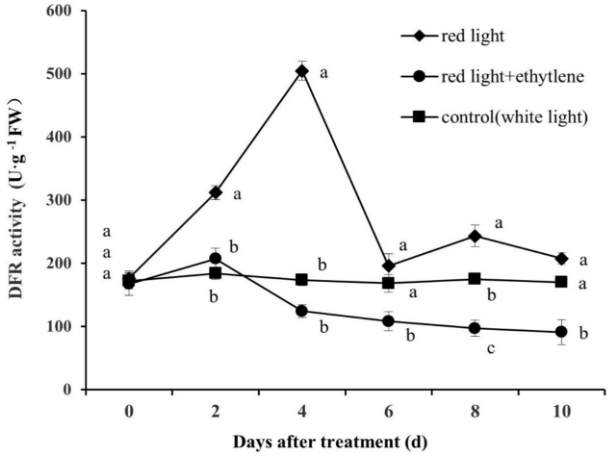

Fig. 6: Changes in dihydroflavnol-4-reductase (DFR) activity in response to red light and/or exogenous ethylene in cabbage leaves. Data are the mean ± SD of 3 replicates. Means followed by different letters at the same time point are significantly different at $P \leqq 0.05$ as determined by Duncan's multiple range test

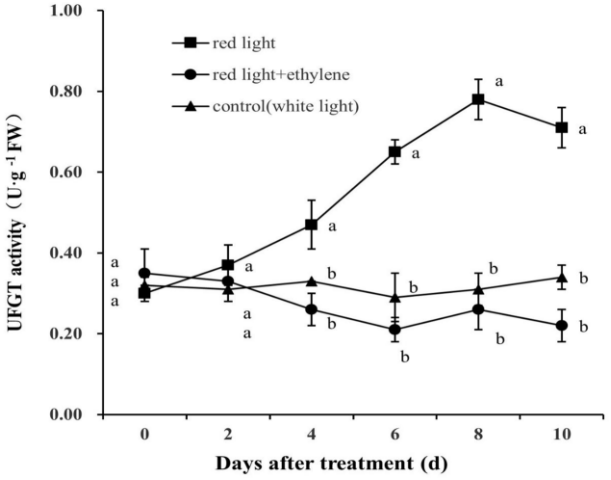

Fig. 7: Interactive effects of red light and exogenous ethylene on flavonoid 3-o-glucosyltransferase (UFGT) in cabbage leaves. Data are the mean ± SD of 3 replicates. Means followed by different letters at the same time point are significantly different at $P \leqq 0.05$ as determined by Duncan's multiple range test

anthocyanin content and the key enzymes responsible for its synthesis was not totally independent, rather it was partly dependent on the external stimuli (red light) and endogenous signal (ethylene).

Discussion

The accumulation of anthocyanins is critically controlled by genetic, hormonal and environmental factors *via* the expression of important structural genes (Stracke *et al*., 2010; Jia *et al*., 2011; Azuma *et al*., 2012; Xie *et al*., 2012; Li *et al*., 2013; Abdullah *et al*., 2018). In the present study, we found that red light enhanced anthocyanin biosynthesis in two cabbage cultivars; however, exogenous ethylene treatment inhibited the anthocyanin concentration under red light conditions (Fig. 2–3). In agreement with Fan *et al*. (2013), we also found an increased biomass accumulation under red and blue light in both cabbage cultivars (Table 1). Biochemical analysis of anthocyanin biosynthetic enzymes revealed that exogenous ethylene-induced attenuation in anthocyanin content under red light was attributed to the suppression of the activity of anthocyanin biosynthetic enzymes, such as PAL, CHI, DFR and UFGT (Fig. 4–7). Furthermore, regression analysis suggested that the relationships between anthocyanin content and some key enzymes involved in anthocyanin biosynthesis are largely dependent on both ethylene and red light in cabbage (Table 2).

Previous studies showed that 2-chloroethylphosphonic acid (ethylene) induced anthocyanin accumulation in peels of apples, plums and strawberries (Gomez-cordoves *et al*., 1996; Bellincontro *et al*., 2006; Villarreal *et al*., 2010; Ma and Wang, 2015). Ethylene can up-regulate the expression of genes involved in anthocyanin synthesis in black carrot roots and grape berries, such as *CHS, F3H, PAL1, PAL3, F3H1, DFR1* and *LDOX2* (El-Kereamy *et al*., 2003; Barba-Espín *et al*., 2017). In anthurium, the anthocyanin content increased, and the expression of genes such as *AnCHI, AnC4H, AnPAL* and *AnANS* up-regulated when treated with 2-chloroethylphosphonic acid (Zheng *et al*., 2018). All these studies showed a positive effect of ethylene on anthocyanin synthesis.

However, our results argued that 2-chloroethylphosphonic acid (ethylene) could inhibit the

synthesis of anthocyanin under red light, meaning that ethylene showed a negative effect on anthocyanin synthesis. These results were inconsistent with the above-mentioned reports, but agreed with Das *et al*. (2012), who showed that ethylene down-regulated the biosynthesis of anthocyanin in *Arabidopsis* (Das *et al*., 2012). Furthermore, the study on tree peony indicated that ethylene inhibited the expression of anthocyanin biosynthesis genes, and resulted in the decreased anthocyanin content (Gao *et al*., 2015). Almasi *et al*. (2012) also showed that ethylene inhibited the synthesis and accumulation of anthocyanin in dendrobium which supports our current findings. Over-expression of ethylene receptor mutant gene (cm-etr1/H69A) in tobacco resulted in increased anthocyanin content in transgenic plants (Takada *et al*., 2005), which further supports the notion that ethylene negatively regulates anthocyanin biosynthesis. As for the mechanism, Jeong *et al*. (2010) inferred that ethylene inhibited anthocyanin synthesis in *Arabidopsis* by down-regulating the expression of bHLH (GL3, TT8, EGL3) and MYB (PAP1, PAP2) transcription factors at the transcriptional level (Jeong *et al*.，2010). Our present results indicated that ethylene inhibited the synthesis of anthocyanins by down-regulating the activity of PAL, CHI, DFR and UFGT.

There are two diametrically opposite views on ethylene-regulated anthocyanin synthesis (Das *et al*., 2012; Gao *et al*., 2015; Barba-Espín *et al*., 2017; Zheng *et al*., 2018). Based on the above discussion, it appears that ethylene-induced differential regulation is closely associated with the differences in plant tissue types as the ethylene-induced positive regulation of anthocyanin synthesis mainly occurred in fruits (Gomez-cordoves *et al*., 1996; Bellincontro *et al*., 2006; Villarreal *et al*., 2010; Ma and Wang, 2015), while the negative regulation mainly found in the vegetative organs such as leaves (Das *et al*., 2012). In line with this, we found a negative effect of ethylene on red light-induced anthocyanin accumulation (Fig. 3). In most cases, the content of anthocyanin in fruits is high, which is positively correlated with the quality of fruits (Abdullah *et al*., 2018). But in case of leafy vegetable, this thumb rule cannot be generalized because excessive anthocyanin accumulation in green cabbage minimizes quality and market value of vegetables. Therefore, ethylene can be used to minimize the reddening of cabbage as an eco-friendly approach. However, future research should be directed towards exploration of the expression pattern of anthocyanin in vegetative and reproductive organs in order to clarify such speculation.

In anthocyanin biosynthesis pathway, PAL catalyzes the dehydrogenation of phenylalanine to form cinnamic acid, which is considered as the rate limiting step of phenylpropanoid biosynthesis (Gu *et al*., 2007). The relationship between PAL activity and plant anthocyanin synthesis has been controversial. Studies by Zhou *et al*. (1997) showed a positive correlation between PAL activity and anthocyanin content in fruits of apple. Results of Lister and Lancaster (1996) pointed out that the PAL activity was closely related to anthocyanin synthesis only when the peel became red. However, Wang *et al*. (2004) found that PAL activity was not related to anthocyanin synthesis in Litchi peel. Our study indicated that there was a positive correlation between anthocyanin content and PAL activity since it showed a quadratic curve under the red light ($y = 0.007 x^2 + 1.145 x 1.145$, $r = 0.67$*) and a linear relationship under the red light plus ethylene treatment ($y=0.207x + 8.708$，$r=0.63$*). Based on the results of this study and some previous reports, we inferred that the relationship between anthocyanin content and PAL activity might be closely associated with the environments, plant developmental stage and species.

Notably, Nakatsuka *et al*. (2008) found a positive correlation between the expression of DFR and anthocyanin synthesis. Similarly, Kim *et al*. (2004) showed that lack of DFR activity in onion inhibited anthocyanin accumulation. However, Murray and Hacket (1991) reported that the DFR activity varied with the developmental stages in ivy. For instance, DFR activity remained high alongside the synthesis of anthocyanins at the young stage, however, after maturation, DFR activity became relatively low and the synthesis of anthocyanins declined with the increased accumulation of quercetin (Murray and Hacket, 1991). Our results showed that the correlation between anthocyanin content and DFR activity was not obvious under red light ($y=0.0001x^2+0.094x+9.004$, $r = 0.62$), while the same was strong under the treatment of red light plus ethylene ($y= -0.001x^2+0.416x-17.057$, $r=0.99$*). Thus, the relationship between anthocyanin content and DFR activity is variable and potentially dependent on environments (such as light and ethylene) and developmental stage of plants.

Conclusion

In the current study, red light significantly increased anthocyanin content in cabbage leaves (Fig. 2). Although the responses slightly differed depending on the cultivars of cabbage as well as light quality, the red light treatment showed a profound effect on anthocyanin content which was largely attributed to transient increases in the activity of key biosynthetic enzymes such as PAL, CHI, DFR and UFGT. Exogenous ethylene treatment significantly suppressed the transient elevation in the activity of those enzymes and abolished the effect of red light on anthocyanin content in a dose-dependent manner (Figs. 3–7). Regression analysis further confirms that the relationships between anthocyanin content and some key enzymes responsible for its synthesis are largely dependent on both ethylene (endogenous signal) and red light (environmental stimuli) in cabbage (Table 2). This study deepens our current knowledge of the light quality-regulated changes in secondary metabolism in cabbage and might be useful for the agronomic management of vegetables.

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