

# Cryptochrome, Phytochrome, and Anthocyanin Production

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## ABSTRACT

Anthocyanin production in cabbage (*Brassica oleracea* L.) and tomato (*Lycopersicon esculentum* Mill.) seedlings exposed to prolonged irradiations was studied under conditions that allowed discrimination, within certain limits, between the contribution of cryptochrome and phytochrome in the photoregulation of the response. The results of the study provide confirming evidence for the involvement of cryptochrome and direct evidence for a significant contribution of cryptochrome to the fluence rate dependence of the response to blue. The results provide some preliminary, direct indication for an interaction between cryptochrome and phytochrome in the photoregulation of anthocyanin production in seedlings exposed to the prolonged irradiations required for a high level of expression of the response. The type and degree of interaction between the two photoreceptors vary significantly, depending on the species and experimental conditions.

Light-dependent anthocyanin production requires prolonged exposures to relatively high fluence rates of visible and near-visible radiation for a high level of response expression (10, 11). The extent of the response is a function of light quality, fluence rate, and exposure duration; action peaks have been found in the UV, BL,<sup>3</sup> R, and FR spectral regions. The relative efficiencies of these regions vary significantly, depending on the species and experimental conditions (10, 11).

Anthocyanin production and other plant photomorphogenic responses to prolonged R and FR irradiations are mediated by phytochrome (10, 11, 16). Responses to UV and BL are mediated by photoreceptors specific for these regions (UV-A/blue-light-photoreceptor, often called cryptochrome, and UV-B-photoreceptor) and/or phytochrome, either inter-

acting or acting independently of one another (2, 7, 19–21, 24).

The unknown nature of the UV-B-photoreceptor and cryptochrome and the fact that UV and BL excite not only these two photoreceptors, but also phytochrome, complicate studies on photoreceptor involvement and interaction in the mediation of responses to UV and BL (7, 14). The selection of light treatments that can be used to discriminate the action of different photoreceptors is based on criteria (18, 24) that take into account the differences between the known (phytochrome) and the inferred (cryptochrome and UV-B-photoreceptor) spectral properties of the photoreceptors.

Both cryptochrome and phytochrome are involved in the photoregulation of anthocyanin production in young seedlings of cabbage (*Brassica oleracea* L.) and tomato (*Lycopersicon esculentum* Mill.) exposed to prolonged irradiations. Phytochrome involvement has been known for a long time (10, 11). Evidence for cryptochrome involvement (25) was obtained recently: BL was found to be significantly more effective than RF (a mixture of R and FR containing no BL) under conditions in which BL and RF maintained the same state of phytochrome in terms of  $\varphi$ ,  $k$ , and  $H$  ( $\varphi_{BL} = \varphi_{RF}$ ,  $k_{BL} = k_{RF}$ , and  $H_{BL} = H_{RF}$ ). Since RF excites phytochrome, but not cryptochrome, and BL excites both photoreceptors, the differences in anthocyanin production between BL and RF treatments that maintain the same state of phytochrome can be reasonably attributed to an involvement of cryptochrome in the mediation of the response to BL (25).

The purpose of the above study (25) was to determine if cryptochrome was involved in the mediation of the response to BL. The methodology used, an application of the principle of equivalent light action (18, 24), was adequate for the purpose, but, as noted (25), was subject to limitations and did not allow for the determination as to whether there was an interaction between cryptochrome and phytochrome, nor the contribution of cryptochrome to the fluence rate dependence of the response to BL.

The purposes of the present study were: (a) to obtain confirming evidence for the involvement of cryptochrome, inasmuch as data obtained with only one experimental approach might not be considered sufficient; (b) to determine whether there is an interaction between cryptochrome and phytochrome in the photoregulation of anthocyanin production under prolonged irradiations; and (c) to determine the contribution of cryptochrome to the fluence rate dependence of the response to BL.

An interaction between the two photoreceptors under the prolonged exposures required for a high level of anthocyanin production has been inferred (10, 11) from data showing a specific cryptochrome-mediated effect of UV-A/BL pretreat-

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<sup>3</sup> Abbreviations: BL, blue; R, red; FR, far red; RF, mixture of R and FR;  $\varphi$ , Pfr/P ratio at photoequilibrium (Pfr/P, ratio between Pfr and total phytochrome;  $k$ , rate constant for phytochrome photoconversion ( $k = k_1 + k_2$ ;  $k_1$  and  $k_2$ , rate constants for Pr to Pfr and Pfr to Pr photoconversions, respectively);  $H$ , light-dependent rate of cycling between Pr and Pfr at photoequilibrium [ $H = (1 - \varphi)k_1 = \varphi k_2 = (\varphi - \varphi^2)k$ ]; BL + R, simultaneous irradiation with BL and R; BL + RF, simultaneous irradiation with BL and RF; BL + FR, simultaneous irradiation with BL and FR;  $N$ , photon fluence rate; cyBL + coR, simultaneous irradiation with cyclic BL and continuous R;  $\Delta R$ , extent of the R-FR reversible response.

ments on the R-FR reversibility of anthocyanin production (sequential interaction [3, 4, 19–22]). Some direct evidence for an interaction between the two photoreceptors in responses to prolonged irradiations has been obtained in studies of stem elongation (1, 6, 8, 19, 24), but there has never been a direct demonstration of an interaction between cryptochrome and phytochrome in the photoregulation of anthocyanin production under the prolonged irradiations required for a high level of expression of the response.

The methodology used in the present study consisted of a comparison of the effects of light treatments differing in the state of only one of the two photoreceptors (same state of phytochrome and different state of cryptochrome, or vice versa). As an example, BL and simultaneous, dichromatic BL + R treatments differed in the state of phytochrome and maintained the same state of cryptochrome; R and BL + R treatments differed in the state of cryptochrome and maintained the same state of phytochrome.

## MATERIALS AND METHODS

### Plant Material

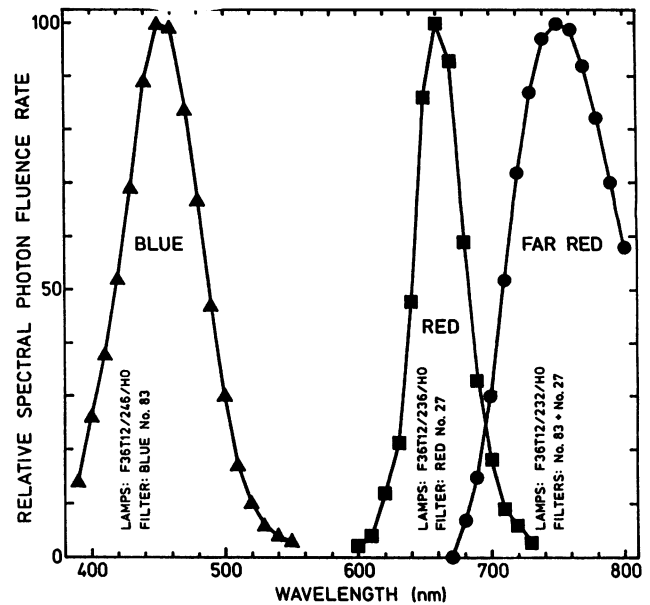
Samples of 30 seeds each of cabbage (*Brassica oleracea* L.; lot F of ref. 25) or 55 seeds of tomato (*Lycopersicon esculentum* Mill.; same lot of ref. 25) were sown in Petri dishes on filter paper moistened with a solution of streptomycin (200  $\mu\text{g/ml}$ ) and incubated in darkness for 4 d at 20 (cabbage) or 25°C (tomato). Streptomycin inhibits Chl synthesis and enhances anthocyanin production (17); the inhibition of Chl synthesis reduces the effects of Chl screening on the state of the photoreceptors during the course of the prolonged exposures. Light treatments were started 4 d after sowing; temperature during the light treatments was 22 to 23°C.

### Extraction and Assay of Anthocyanin

Samples of 30 (cabbage) and 55 (tomato) seedlings each were extracted (2 d at 3–5°C, with shaking) with acidic methanol (1% HCl, w/v). The *A* of the extracts, clarified by filtration, was measured at 530 nm (peak of absorption of anthocyanin) and 657 nm (peak of absorption of Chl in acidic methanol; the absorption of Chl in acidic methanol at 530 nm is 24–26% of that at 657 nm). The formula,  $A_{530} - 0.25 A_{657}$ , was used to compensate for the contribution of Chl to the  $A_{530}$ , even though the contribution was small because of the reduction of Chl synthesis by streptomycin. Each *A* value reported in the Figures and Tables is a mean value from a minimum of 16 replicate samples; the SES were about 3 to 5% of the mean values.

### Light Sources

The BL, R, and FR sources (Fig. 1) were mounted on a steel frame in a temperature-controlled room; RF light was obtained by simultaneous irradiation with R and FR ( $N_R/N_{FR} = 0.2$ ). Neutral density filters (black polypropylene fabrics of various mesh sizes; transmission curves with 2 to 3% maximum deviation between 350 and 800 nm) were used when required. Light measurements were taken with an ISCO-SR spectroradiometer.



**Figure 1.** Spectral photon fluence rate distribution of the BL, R, and FR light sources. Fluorescent lamps (type F36T12/HO) used: Sylvania special phosphor nos. 246 (BL), 236 (R), and 232 (FR). Roscolux filters used: nos. 83 (BL), 27 (R), and 83 plus 27 (FR).

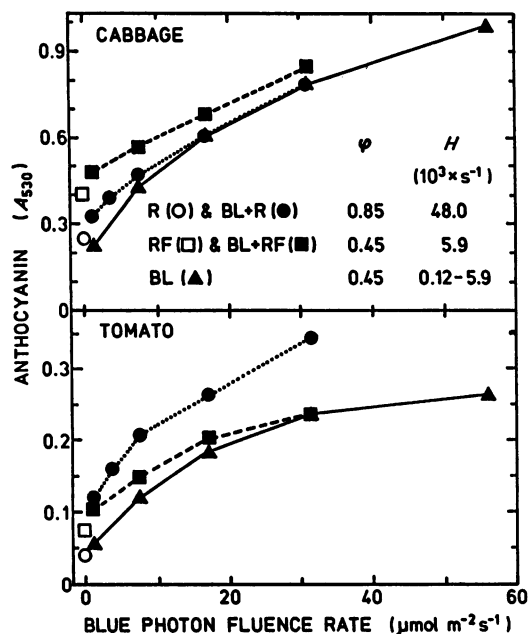
The measured *in vivo* values of  $\phi$  in seedlings exposed to light from the above sources were:  $\phi_R = 0.85 \pm 0.02$ ;  $\phi_{BL} = 0.45 \pm 0.03$ ;  $\phi_{RF} = 0.45 \pm 0.04$ ;  $\phi_{FR} = 0.13 \pm 0.01$ .

The measured R/BL effectiveness ratio for phytochrome photoconversion *in vivo* was about 40, close to previously reported values (12); thus, it was possible to add relatively high BL fluence rates to R without causing large changes in the state of phytochrome. For the treatments in Figures 2 and 3,  $N_R$  was  $25 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; the measured values of  $\phi$  *in vivo* were  $0.85 \pm 0.02$  under R and  $0.84 \pm 0.03$  under BL + R at the maximum  $N_{BL}$  ( $32 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) added to R; *H* varied from  $0.047 \pm 0.003 \text{ s}^{-1}$  under R to  $0.051 \pm 0.004 \text{ s}^{-1}$  under BL + R at the maximum  $N_{BL}$  added to R; the *H* value of  $0.048 \text{ s}^{-1}$  shown in Figure 2 for R and BL + R is an average value.

The measured RF/BL effectiveness ratio for phytochrome photoconversion *in vivo* was about 11. The dichromatic BL + RF treatments ( $\phi_{BL+RF} = \phi_{BL} = \phi_{RF} = 0.45$ ) were carried out at a value of *H* ( $H_{BL+RF} = 5.9 \times 10^{-3} \text{ s}^{-1}$ ) equal to those produced under BL at  $N_{BL} = 57 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$ , and under RF at  $N_{RF} = 5.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Maintaining a constant value of *H* under BL + RF (Fig. 2) required simultaneous adjustments of  $N_{RF}$  (decrease from 5.1–2.3  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and  $N_{BL}$  (increase from 1.2–32  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

### Spectrophotometric Assay of Phytochrome *in Vivo*

A computer-controlled dual-wavelength spectrophotometer, optimized for phytochrome measurements (23), was used for the *in vivo* assays. Measurements of  $\phi$  and *k* were carried out for each light treatment, following a protocol described in detail previously (12). Rates of cycling cannot be measured directly and were calculated from the measured values of  $\phi$  and *k* according to the equation,  $H = (\phi - \phi^2)k$ .



**Figure 2.** Anthocyanin production in cabbage and tomato seedlings exposed for 24 h to continuous BL, R, RF, BL + R, and BL + RF treatments. The  $A_{530}$  values reported were corrected by subtracting the dark-control values (0.046 for cabbage and 0.014 for tomato).  $N_{BL}$  in BL (▲), BL + R (●), and BL + RF (■), as indicated.  $N_R$  in R (○) and BL + R (●),  $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ .  $N_{RF}$  in RF (□),  $5.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; in BL + RF (■), decreasing from  $5.1$  to  $2.3 \mu\text{mol m}^{-2} \text{s}^{-1}$  as  $N_{BL}$  increased from  $1.2$  to  $32 \mu\text{mol m}^{-2} \text{s}^{-1}$ . At the  $N_{RF}$  used in BL + RF, anthocyanin production under RF only decreased with decreasing  $N_{RF}$  from  $0.345$  to  $0.255$  in cabbage and from  $0.074$  to  $0.062$  in tomato.

Values of  $\varphi$  and  $H$  from *in vivo* assay were preferred over projected values, calculated from photoconversion cross-sections of purified phytochrome (*e.g.* [9]) and the spectral photon fluence rate distribution of the light sources used. Projected values vary significantly, depending on the particular set of photoconversion cross-sections used in the calculations (12, 13) and are significantly different from those measured *in vivo* (12). For example, for the light sources used in this study (Fig. 1), the projected R/BL effectiveness ratio for phytochrome photoconversion was about 10 to 12, but it was about 40 according to the *in vivo* measurements (see also ref. 12); the measured, *in vivo* value of  $\varphi_{RF}$  was 0.45; projected values of  $\varphi_{RF}$  varied from 0.27 to 0.35. The differences between projected and measured *in vivo* values are significant and probably due to differences among the geometry, optical properties, and phytochrome distribution in solution and in tissues.

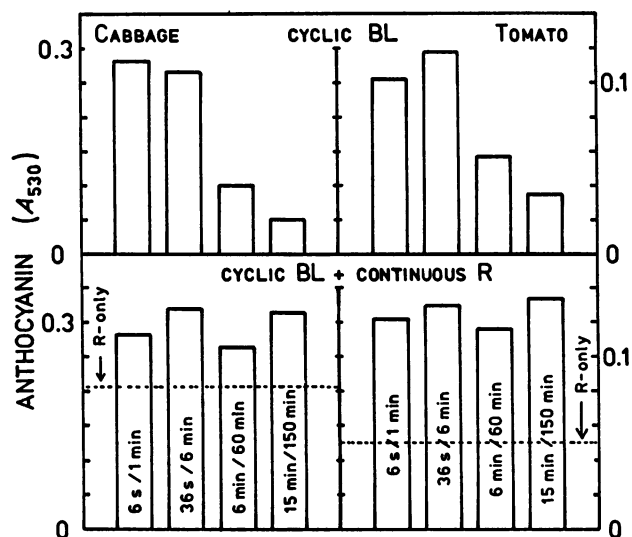
Even though values of  $\varphi$  and  $H$  from *in vivo* assays were used, there are still some limitations: these values were measured in 4-d-old, dark-grown seedlings, and might vary during the prolonged irradiations. Within a few hours after the onset of light, as a consequence of phytochrome destruction, the phytochrome level becomes too low for accurate measurements of photoconversion kinetics, especially in tomato, whose initial phytochrome content ( $0.007$ – $0.009 \Delta\Delta A/40$  seedlings) is much lower than in cabbage ( $0.020$ – $0.024 \Delta\Delta A/$

25 seedlings). Values of  $\varphi$ , measured about 3 h after the onset of light, were not significantly different from those reported. But  $\varphi$  is only one parameter and, even assuming that it might remain constant throughout the course of a 24-h light treatment, [Pfr] would decrease as a consequence of the decrease of the level of phytochrome (*e.g.* in cabbage, 6 h after the onset of the light treatment, the  $\Delta\Delta A$  was  $0.003$ – $0.005/25$  seedlings). Despite these limitations, values of  $\varphi$  and  $H$  from *in vivo* measurements should still provide a more reliable estimate of the state of phytochrome in the seedlings than projected values, at least for the first few hours of the light treatments.

## RESULTS

The selection of simultaneous, dichromatic BL + R and BL + RF treatments was based on the results of a study on the effects of light quality and fluence rate on phytochrome-mediated anthocyanin production in cabbage and tomato seedlings, under conditions in which phytochrome was the only photomorphogenic photoreceptor excited (15). Dichromatic BL + R was selected because of the low effectiveness of R for anthocyanin production (15), and BL + RF for a comparison between treatments maintaining the same value of  $\varphi$ :  $\varphi_{BL} = \varphi_{RF} = \varphi_{BL+RF} = 0.45$ .

In both species, under continuous irradiations (Fig. 2), anthocyanin production under BL, at the maximum  $N_{BL}$  ( $57 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) used, was much higher than under RF ( $N_{RF} = 5.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), which maintained the same state of phytochrome ( $\varphi_{BL} = \varphi_{RF} = 0.45$ ;  $H_{BL} = H_{RF} = 5.9 \times 10^{-3} \text{s}^{-1}$ ), and R, which maintained higher values of  $\varphi$  and  $H$  ( $\varphi_R =$



**Figure 3.** Anthocyanin production in cabbage and tomato seedlings exposed for 25 h to cyclic BL applied either alone or simultaneously with continuous R. The  $A_{530}$  values were corrected by subtracting the dark control values (0.048 for cabbage and 0.015 for tomato). Fluence rates:  $N_{BL}$ ,  $31 \mu\text{mol m}^{-2} \text{s}^{-1}$ ;  $N_R$ ,  $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Numbers within bars, length of exposure to BL/length of BL cycle. Under cyclic BL, Pfr/P decreases during the dark interval between successive exposures to BL; after a saturating exposure to BL, Pfr/P decreased in darkness from 0.45 to 0.27 in 55 min to 0.14 in 135 min.

0.85;  $H_R = 4.8 \times 10^{-2} \text{ s}^{-1}$ ). The differences in the extent of the response between BL and RF (for  $\varphi_{BL} = \varphi_{RF}$  and  $H_{BL} = H_{RF}$ ) suggest that the contribution of cryptochrome to anthocyanin production under BL may be about 60% of the total response to BL in cabbage and 70% in tomato. Differences in the extent of the response between treatments differing only in the state of cryptochrome (e.g. RF and BL, R and BL + R, RF and BL + RF) provide evidence for an involvement of cryptochrome in the photoregulation of anthocyanin production.

The continuous BL + R and BL + RF treatments (Fig. 2) were carried out under conditions maintaining constant values of  $\varphi$  and  $H$ , independently of  $N_{BL}$ . Under these conditions, if phytochrome had been the only photoreceptor involved, anthocyanin production under BL + R and BL + RF should have been independent of  $N_{BL}$ . This was clearly not the case: anthocyanin production under BL + R and BL + RF is a function of  $N_{BL}$  (Fig. 2). These results provide further evidence for the involvement of cryptochrome.

It is not possible to determine the relative contribution of cryptochrome and phytochrome to the fluence rate dependence of responses to BL using BL-only treatments because the states of both photoreceptors change as a function of  $N_{BL}$ . The state of phytochrome ( $\varphi$  and  $H$ ) was independent of  $N_{BL}$  under BL + R and BL + RF. If the contribution of cryptochrome had been small compared with that of phytochrome, anthocyanin production under BL + R and BL + RF should have shown a dependence on  $N_{BL}$  significantly smaller than under BL. This was clearly not the case (Fig. 2), and the results suggest a significant contribution of cryptochrome to the fluence rate dependence of the response to BL.

In cabbage, under continuous irradiation (Fig. 2), the differences in anthocyanin production, small to nil between BL and BL + R, and large to small between BL and BL + RF, decrease with increasing  $N_{BL}$ , whereas the differences in anthocyanin production between R and BL + R and between RF and BL + RF increase with increasing  $N_{BL}$ . The combined

**Table II. Anthocyanin Production in Cabbage and Tomato Seedlings Exposed for 24 h to Continuous BL, R, FR, and Dichromatic BL + R and BL + FR Treatments**

Light treatments started 96 h after sowing;  $N_{BL} = N_R = N_{FR} = 10 \mu\text{mol m}^{-2} \text{ s}^{-1}$ .

Light Treatment	$\varphi^a$	Anthocyanin Produced in	
		Cabbage	Tomato
		$A_{530}^b$	
BL	0.45	0.472	0.139
R	0.85	0.190	0.045
FR	0.13	0.952	0.035
BL + R	0.85	0.508	0.198
BL + FR	0.17	1.237	0.083

<sup>a</sup> Values measured *in vivo*. <sup>b</sup> Corrected by subtraction of dark control values: 0.047 for cabbage and 0.016 for tomato.

effects of BL plus R, and BL plus RF are either less than additive or about additive (Table I), depending on the method used to calculate the additiveness of the effects. Even though the differences are small, BL + RF is more effective than BL + R (Fig. 2). This result seems reasonable for cabbage, a system in which phytochrome-mediated anthocyanin production increases with decreasing  $\varphi$  (15); this suggestion is confirmed by data showing that BL + FR is more effective than BL + R (Table II). Apparently, within the range of  $\varphi$  values (0.13–0.85) used in this study and a previous one (15), anthocyanin production in cabbage, under prolonged irradiations, is dependent on low values of  $\varphi$ , either in the absence or presence of BL (Table II).

In tomato, under continuous irradiation (Fig. 2), the differences in anthocyanin production between BL and BL + RF decrease with increasing  $N_{BL}$ ; the differences between RF and BL + RF increase with increasing  $N_{BL}$ . The combined effects of BL plus RF are either less than additive or about additive

**Table I. Comparison of Anthocyanin Production under Dichromatic Continuous BL + R and BL + RF and cyBL + coR with Projected Productions Calculated Assuming Additivity of the Effects of BL and R and BL and RF**

$\Delta\% = 100 \times (m - p)/p$ , where  $m$  is anthocyanin production under dichromatic treatments,  $p$  is projected value, calculated for  $\Delta\%(A)$  as the sum of anthocyanin production under BL and R or RF, and for  $\Delta\%(B)$  as the sum of anthocyanin production under R or RF and the portion of the response to BL attributed to cryptochrome action (60% in cabbage and 70% in tomato; see "Results"). Limitations in the calculations of  $p$ : the first method does not take into account that a portion of the response to BL might be due to phytochrome only; the second method does not take into account that a change in the state of one photoreceptor might affect the action of the other.

	Cabbage				Tomato			
	BL + R		BL + RF		BL + R		BL + RF	
	$\Delta\%(A)$	$\Delta\%(B)$	$\Delta\%(A)$	$\Delta\%(B)$	$\Delta\%(A)$	$\Delta\%(B)$	$\Delta\%(A)$	$\Delta\%(B)$
Continuous irradiations (BL + R and BL + RF)								
$N_{BL} (\mu\text{mol m}^{-2} \text{ s}^{-1})$								
1.2	-32	-16	-21	-7	27	53	-21	-10
3.1	-29	-9			35	67		
7.9	-31	-8	-28	-8	28	65	-24	-6
17.5	-30	-3	-27	-1	18	57	-20	2
32.2	-25	8	-21	12	26	69	-20	5
cyBL + coR (150 min BL cycle)								
	22	33			56	77		

(Table I), depending on the method used to calculate the additiveness of the effects. The differences in anthocyanin production between BL and BL + R and between R and BL + R are larger than expected, assuming additivity of the effects of BL and R (Table I); this observation suggests a synergistic interaction between cryptochrome and phytochrome. Anthocyanin production is higher under BL + R than BL and BL + RF; this result suggests that, in tomato, values of  $\varphi$  and/or  $H$  higher than those established under BL and BL + RF might be required for a high level of expression of the cryptochrome-mediated effects of BL. This suggestion is further supported by data showing that, in tomato, under prolonged irradiations, anthocyanin production is significantly lower under BL + FR than BL + R (Table II).

Under cyclic BL (Fig. 3), differences in the state of phytochrome between cycles of different duration might be the main cause for the decrease in anthocyanin production with increasing cycle duration (increasing dark interval between successive BL exposures; Pfr/P decreases during the dark interval). This suggestion is based on the observation that the duration of the BL cycle has no significant effect on anthocyanin production under cyBL + coR treatments; the latter maintain constant values of  $\varphi$  and  $H$ . Significant differences in anthocyanin production between cyclic BL and cyBL + coR were observed for BL cycles of long (60 and 150 min), but not short (1 and 6 min) duration. Synergistic effects between cyBL and coR were observed only for the longest BL cycle (150 min) used in cyBL + coR treatments. The lack of an effect of the duration of the BL cycle under cyBL + coR treatments suggests that the cryptochrome-mediated effects of BL on the responding system might be relatively long-lived. This observation is consistent with data showing that the cryptochrome-mediated effects of BL pretreatments decay with a half-life of about 6 h in milo (*Sorghum vulgare* Pers.) seedlings (4).

The extent of the R-FR reversible response ( $\Delta R$ ) for anthocyanin production in seedlings not previously exposed to light is minimal in cabbage and nil in tomato and is significantly enhanced by light pretreatments (Table III). These results are consistent with previous findings (3–5, 10, 11, 19–22). Blue pretreatments are more effective than R and FR ones in increasing  $\Delta R$  (Table III), another observation consistent with previous findings (3–5, 19–22). The effects of BL pretreatments on  $\Delta R$  are enhanced by adding R and reduced by adding FR during the pretreatments (Table III). These results are different from those reported for anthocyanin production in milo seedlings, a system in which the effects of BL pretreatments on  $\Delta R$  are not modified by adding FR to BL during the pretreatment (4). These differences are not surprising in consideration of the well-documented variability in the effects of light on anthocyanin production, depending on the species and experimental conditions (10, 11, 20, 21). To the best of our knowledge, insofar as anthocyanin production is concerned, milo is the only system in which a previous attempt (4) was made to determine the effects of changes in the state of phytochrome during the BL pretreatment on the cryptochrome-mediated effects of BL on  $\Delta R$ .

## DISCUSSION

The techniques that can be applied at present to studies on photoreceptors' involvement and interaction in the mediation

**Table III.** Effect of Light Pretreatments on the R-FR Reversibility of Anthocyanin Production

Light treatments were started 96 h after sowing; schedule of treatments: 8 h pretreatment → 5 min R or 5 min FR → 24 h dark. The light sources used for the pretreatments were the same as in Table II.

Pretreatment and Species	Anthocyanin at End of Pretreatment	Anthocyanin Produced during 24 h Dark <sup>a</sup> after Pretreatments Terminated by		$\Delta R^b$
		5 min R	5 min FR <sup>c</sup>	
		$A_{530}$	$A_{530}$	
<b>Cabbage</b>				
Dark	0.048	0.035	0.016	0.019
BL	0.103	0.267	0.115	0.152
R	0.077	0.156	0.058	0.098
FR	0.137	0.404	0.311	0.093
BL + R	0.090	0.338	0.146	0.192
BL + FR	0.180	0.529	0.411	0.118
<b>Tomato</b>				
Dark	0.016	0.002	0.000	0.002
BL	0.019	0.067	0.032	0.035
R	0.016	0.035	0.011	0.024
FR	0.016	0.032	0.012	0.020
BL + R	0.018	0.091	0.031	0.060
BL + FR	0.019	0.038	0.017	0.021

<sup>a</sup> Anthocyanin production during the 24-h dark after the pretreatments is the difference between total anthocyanin production (pretreatment → 5 min R or FR → 24 h dark) and anthocyanin production during the 8 h pretreatment. <sup>b</sup>  $\Delta R$  is the extent of the R-FR reversible response: difference in anthocyanin production between "pretreatment → 5 min R → 24 h dark" and "pretreatment → 5 min FR → 24 h dark." <sup>c</sup> The FR source used for the terminal 5 min FR consisted of incandescent lamps, water filter, and FRF-700 Plexiglas and Roscolux No. 27 medium red color filters; this source produced a measured *in vivo* value of  $\varphi = 0.02-0.04$ ; an exposure of 2 min was sufficient to establish this value of  $\varphi$ .

of responses to BL are subject to several limitations. The nature of these limitations and their consequences on the interpretation of the experimental results have been analyzed in detail (7), and there is no need for repetition. The interpretation of the results of the present study took into account the limitations of the experimental techniques used.

The results (Figs. 2 and 3) provide confirming evidence for the involvement of cryptochrome in the photoregulation of anthocyanin production in cabbage and tomato seedlings. The second objective of this study was to determine the contribution of cryptochrome to the fluence rate dependence of anthocyanin production under continuous BL. The results (Fig. 2) suggest a significant contribution of cryptochrome to the fluence rate dependence of the response. The supporting results and arguments for cryptochrome involvement and cryptochrome contribution to the fluence rate dependence of the response have been discussed (paragraphs 2–4 in "Results"), and there is no need for repetition.

The third objective of this study was to determine whether there was an interaction between cryptochrome and phyto-

chrome in the photoregulation of anthocyanin production in seedlings exposed to the prolonged irradiations required for a high level of expression of the response. As noted in the "Introduction," there has been no previous, direct verification of this particular aspect.

Current hypotheses on photoreceptor interactions in photomorphogenesis (7, 19, 20, 24) suggest that: (a) Pfr might be required for the expression of the cryptochrome-mediated effects of BL, thus, the state of phytochrome might be a limiting factor for the expression of the cryptochrome-mediated effects of BL; and (b) cryptochrome might act by establishing or enhancing, depending on the response-species combination, the sensitivity of the responding system to Pfr. These two suggestions are not mutually exclusive and both consider Pfr as the effector for the expression of the response. There are many results in support of an interaction between cryptochrome and phytochrome, but, at the same time, the data available at present do not allow for the exclusion of an independent action of the two photoreceptors (24). The mechanism of interaction at the molecular level is unknown at present.

The observed differences in anthocyanin production between treatments that maintained the same state of cryptochrome and different states of phytochrome (Fig. 2: BL and BL + R at equal  $N_{BL}$ , BL and BL + RF at equal  $N_{BL}$ , BL + R and BL + RF at equal  $N_{BL}$ ; Fig. 3: cyBL and cyBL + coR with the same BL cycle duration) are consistent with the first of the two suggestions above. The observed differences in anthocyanin production between treatments that maintained the same state of phytochrome and different states of cryptochrome (e.g. Fig. 2: BL + R at different  $N_{BL}$ , BL + RF at different  $N_{BL}$ , R and BL + R, RF and BL + RF) are consistent with the second of the two suggestions above. Essentially, the results obtained in this study show an overall pattern consistent with current hypotheses and provide at least a preliminary, direct indication for an interaction between cryptochrome and phytochrome in the photoregulation of anthocyanin production in cabbage and tomato seedlings exposed to prolonged irradiations.

The type and degree of interaction vary significantly, depending on the species and experimental conditions. In both species, under cyBL + coR (150 min BL cycle; Fig. 3), the combined effects of BL and R are synergistic (Table I). In both species, under continuous BL + RF (Fig. 2), the combined effects of BL and RF are either less than additive or about additive (Table I), depending on the method used to calculate the additivity of the effects. Under continuous BL + R (Fig. 2), the combined effects of BL and R are synergistic in tomato, but are either less than additive or about additive in cabbage (Table I). A synergistic effect is generally considered as an indication of interaction (1, 7, 24); an effect that is less than additive might also suggest an interaction, but an additive effect is generally considered as an indication of an independent action of the two photoreceptors. At least in part, the interpretation of the results is limited by the method used to calculate the additivity of the effects (Table I).

On the basis of the results and the above considerations, one may conclude that there is some evidence for an interaction between the two photoreceptors in the photoregulation of anthocyanin production under the prolonged irradiations

required for a high level of expression of the response. The degree of interaction is apparently more pronounced in tomato than cabbage and, in both species, the type and degree of interaction vary depending on the experimental conditions. These conclusions are limited to the range of experimental conditions used in the study and are subject to the limitations of the experimental techniques used.

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