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Cryptochrome, Phytochrome, and the Photoregulation of Anthocyanin Production under Blue Light

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ABSTRACT

The principle of equivalent light action predicts that two light treatments (wavelengths λ_1 and λ_2) producing the same Pfr/P ratio ($\varphi_{\lambda 1} = \varphi_{\lambda 2}$) and the same rate of phytochrome photoconversion ($k_{\lambda 1} = k_{\lambda 2}$) are perceived by phytochrome as being the same and should produce the same effect. The results of experiments based on the principle of equivalent light action indicate that cryptochrome is involved in the photoregulation of anthocyanin production elicited by blue light in tomato seedlings. This was also the case for one strain of cabbage seedlings. For another strain of cabbage seedlings, the results suggest that cryptochrome is either not involved or that the state of phytochrome is the principal limiting factor.

Light-dependent anthocyanin production in young seedlings displays the characteristics of HIR[3] responses (5). The spectral sensitivity of anthocyanin production varies in different species or strain of the same species (5, 6). In some, FR, R, BL, and UV are all effective; in others, R, BL, and UV are effective, while FR is either ineffective or only minimally effective; in others still, only BL and UV are effective. BL is effective in most of the biological systems studied. Phytochrome is known to be one of the photoreceptors involved in the photoregulation of anthocyanin production, and in some species it may be the only photoreceptor involved (5). However, the results of several studies (9, 11) suggest that another photoreceptor, specific for the UV-BL region of the spectrum and generally known as cryptochrome, might also be involved. The nature of cryptochrome is still unknown: flavin derivatives have been suggested as one possibility (1). There might be more than one cryptochrome (1). In this paper the term cryptochrome is used in general terms, to indicate the photoreceptor(s) specific for the UV-BL region. It is difficult to prove the action of cryptochrome in responses where phytochrome is also active. There is no specific test for cryptochrome. The absorption spectrum of phytochrome (13) shows absorption bands in the UV, BL, R, and FR regions. Thus, responses to BL may be mediated by cryptochrome, phytochrome, or both. In addition, even in those cases in which the action of BL seems to be mediated exclusively through cryptochrome, the final expression of the response might be affected by the state of the phytochrome system (9).

The "light-equivalent principle" predicts that two light treatments (wavelengths λ_1 and λ_2) producing the same value of the Pfr/P ratio at photoequilibrium ($\varphi_{\lambda 1} = \varphi_{\lambda 2}$) and the same rate of phytochrome photoconversion ($k_{\lambda 1} = k_{\lambda 2}$) will be perceived by phytochrome as being the same and should produce the same effect (11, 12). According to the light-equivalent principle, the state of phytochrome under BL (*e.g.* 440–460 nm, absorbed by both cryptochrome and phytochrome) should be the same as that under exposure to light of wavelengths between 690 and 700 nm (absorbed by phytochrome, but not by cryptochrome), with about one-tenth of the photon fluence rate of BL. Under these conditions, if the effects produced by BL (440–460 nm) and 690 to 700 nm irradiations on a given response are quantitatively different, one can conclude that cryptochrome is involved in the photoregulation of the response elicited by BL.

We carried out experiments based on the principle of equivalent light action to determine the involvement of cryptochrome in the mediation of the action of BL on anthocyanin production in cabbage and tomato seedlings.

MATERIALS AND METHODS

Plant Material. Seeds of tomato (*Lycopersicon esculentum* Mill., cv Beefsteak) and two strains (D and F) of cabbage (*Brassica oleracea* L., cv Red Acre), obtained from the W. A. Burpee Co., Warminster, PA, were sown in Petri dishes on filter paper (Whatman No. 3) moistened with a solution of streptomycin (200 μg/ml). Streptomycin inhibits the development of the chloroplast and the synthesis of Chl and enhances anthocyanin production (8). Streptomycin was used primarily to reduce the effects of Chl screening on the state of phytochrome in the tissue. The light treatments were started 72 h after sowing for cabbage and 96 h after sowing for tomato. Temperature throughout the experimental period was 22 to 23°C.

Extraction of Anthocyanin. Lots of 30 seedlings each for cabbage and 70 seedlings for tomato were extracted with acidified (1% HCl, w/v) methanol for 2 d, at 3 to 5°C, with occasional shaking. Extracts were clarified by filtration and the A was measured at 530 and 657 nm, as described previously (7). Mean values (±SE) reported in the figures were calculated from a minimum of four replicates for each of the light treatments after correction by subtraction of the values of the dark controls.

Light Sources. The BL source was a Schoeffel 2.5 kw xenon-arc with a 10 cm water filter and a wide bandpass blue filter. The long wavelength source (RF) was a Withrow 1.5 kW incandescent lamp source with a 10 cm water filter and a cutoff red filter (Corning 2-64). The spectral energy distribution (Fig. 1) of the light sources was measured with a Gamma model C-3 spectro-

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[3] Abbreviations: HIR, high irradiance reaction; BL, blue light; R, red light; FR, far red light; RF, 650 to 800 nm light; P, total phytochrome, Pr + Pfr; φ, Pfr/P ratio at photoequilibrium; σ, phytochrome photoconversion cross-section; $k = \sigma N$, rate constant for phytochrome photoconversion; N, photon fluence rate.

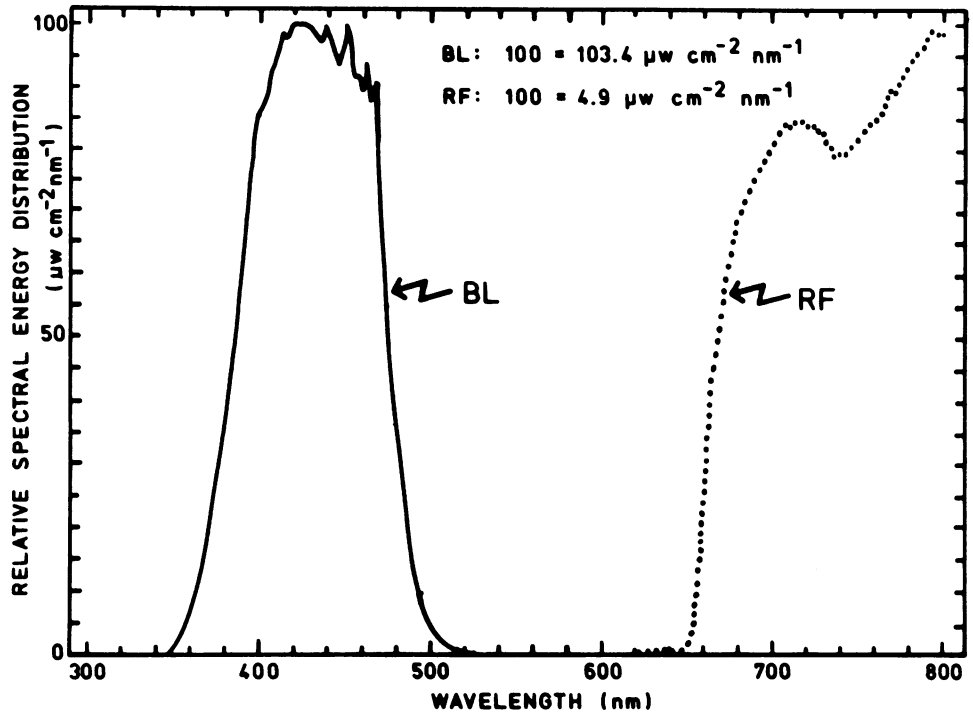

FIG. 1. Spectral energy distribution of BL and RF light sources.

radiometer (Gamma Scientific, San Diego, CA). Two problems made it impossible to use narrow-band interference filters. First, despite the rather extensive collection of interference filters at the two institutions, we could not find narrow-band interference filters for the BL and the long wavelength regions that would produce Pfr/P ratios at photoequilibrium matched within 5% of each other. Second, the maximum output of the xenon-arc source with a narrow band (10 nm half-bandwidth) 450 nm interference filter over a uniform 10 cm diameter field was less than 10 μE m^{-2} s^{-1}, about one order of magnitude lower than that required for the experiments. Some problems with the water supply used to cool the light sources prevented us from carrying out irradiations longer than 10 h.

Phytochrome Assay. A custom-built, microcomputer-controlled dual-wavelength spectrophotometer (10) was used to determine total phytochrome content, values of Pfr/P ratios at photoequilibrium and rates of phytochrome photoconversion *in vivo* under BL and RF irradiations in both cabbage and tomato seedlings. The sample size used for these measurements was 50 seedlings packed in a 1-cm diameter cuvette. As a reference standard for the Pfr/P ratio at photoequilibrium under red light we used the value of 0.86 (4, 14).

The values of the *in vivo* Pfr/P photoequilibrium ratios established under BL and RF irradiations were:

$$\varphi_{BL} = 0.56 \pm 0.03; \varphi_{RF} = 0.57 \pm 0.03$$

The rate constant for phytochrome photoconversion, k, can be calculated from the equation $k = (\ln 2)/t^{1}/_{2}$, where $t^{1}/_{2}$ is the duration of the irradiation required to reach 50% of the value of the Pfr/Pr ratio at photoequilibrium. The apparent phytochrome photoconversion cross-sections *in vivo* under BL and RF can be calculated from the relation $\sigma = k/N$; they were:

$$\sigma_{BL} = 2.39 \times 10^{-4} \text{ m}^2 \text{ } \mu\text{E}^{-1}; \sigma_{RF} = 2.12 \times 10^{-3} \text{ m}^2 \text{ } \mu\text{E}^{-1}$$

Since the value of the σ_{RF}/σ_{BL} ratio is 8.87, equal rates of phytochrome photoconversion under exposures to BL and RF ($k_{BL} = k_{RF}$) are obtained when $N_{BL} = 8.87N_{RF}$.

It is important to emphasize the fact that the values used for φ and σ were not theoretical values, calculated on the basis of the spectral energy distribution of the BL and RF sources (Fig. 1) and known *in vitro* spectral parameters of phytochrome (4, 14), but were instead those actually measured *in vivo* in the seedlings used for the experiments. A summary of the experimental conditions is given in Table I.

RESULTS AND DISCUSSION

In tomato, when anthocyanin production is expressed as a function of the rate of phytochrome photoconversion (rate constant k, Fig. 2a), BL is significantly more effective than RF. In the range of overlapping photon fluence rates (Fig. 3a), BL and RF are equally effective, even though BL is much less effective than RF for phytochrome photoconversion. These results indicate that cryptochrome is involved in the mediation of the action of BL, and support previous suggestions (2, 3, 9) for the involvement of cryptochrome in the mediation of BL action on anthocyanin production in tomato seedlings.

In cabbage seedlings of strain D, when anthocyanin production is expressed as a function of k (Fig. 2b), there is no significant difference between BL and RF. If anthocyanin production is expressed as a function of the photon fluence rate (Fig. 3b), RF is significantly more effective than BL. About 20 μE m^{-2} s^{-1} of BL and 3 μE m^{-2} s^{-1} of RF are required for the production of the same amount ($A_{530} = 0.5$) of anthocyanin; thus, in terms of quantum efficiency, RF is about seven times as effective as BL;

Table I. *Summary of Experimental Conditions for the Experiments of Figures 2 and 3*

N_{BL}	N_{RF}	k[a]	$t^{1/2}$[b]
$\mu E\ m^{-2}\ s^{-1}$		s^{-1}	s
3.02	0.34	7.2×10^{-4}	960
9.20	1.03	2.2×10^{-3}	317
30.2	3.40	7.2×10^{-3}	96
92.0	10.3	2.2×10^{-2}	31.7
302.0	34.0	7.2×10^{-2}	9.6

[a] Rate constant for phytochrome photoconversion: $k = k_{BL} = k_{RF}$. [b] Exposure time required to reach 50% (0.5 φ) of the Pfr/P ratio at photoequilibrium; $\varphi_{BL} = \varphi_{RF} = 0.56$–$0.57$.

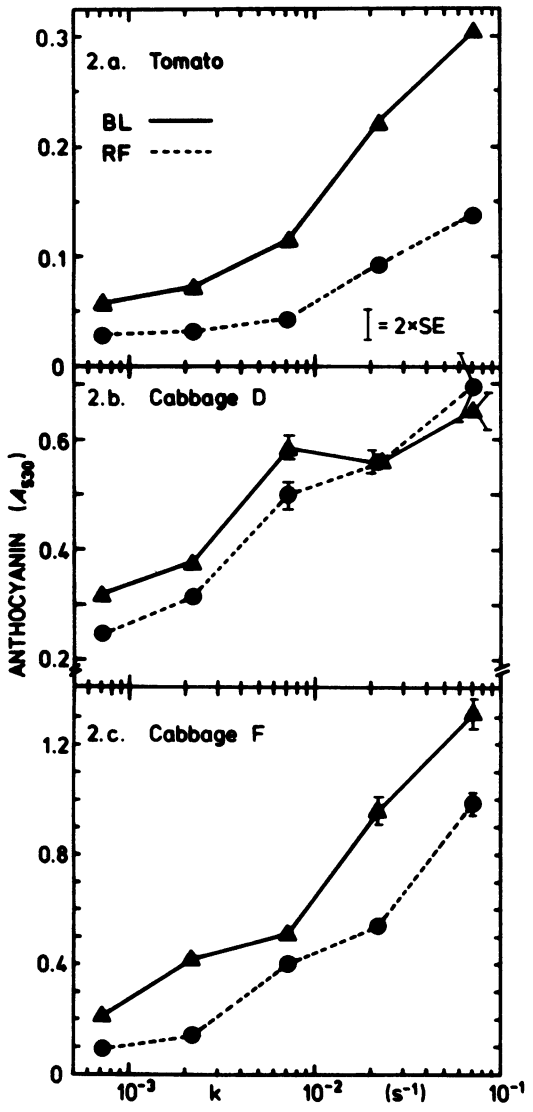

FIG. 2. Anthocyanin production under BL and RF irradiations as a function of k, the rate constant of phytochrome photoconversion. Light treatments: tomato, 96 h D + 2 × (8 h BL or RF + 16 h D); cabbage D, 72 h D + 8 h BL or RF + 16 h D; cabbage F, 72 h D + 2 × (8 h BL or RF + 16 h D). Absorbance values corrected by subtraction of the values of the dark controls (tomato, 0.02; cabbage D, 0.31; cabbage F, 0.06). When not reported, 2 × SE values are equal to or less than the dimensions of the circle and triangle symbols. Other details in Table I.

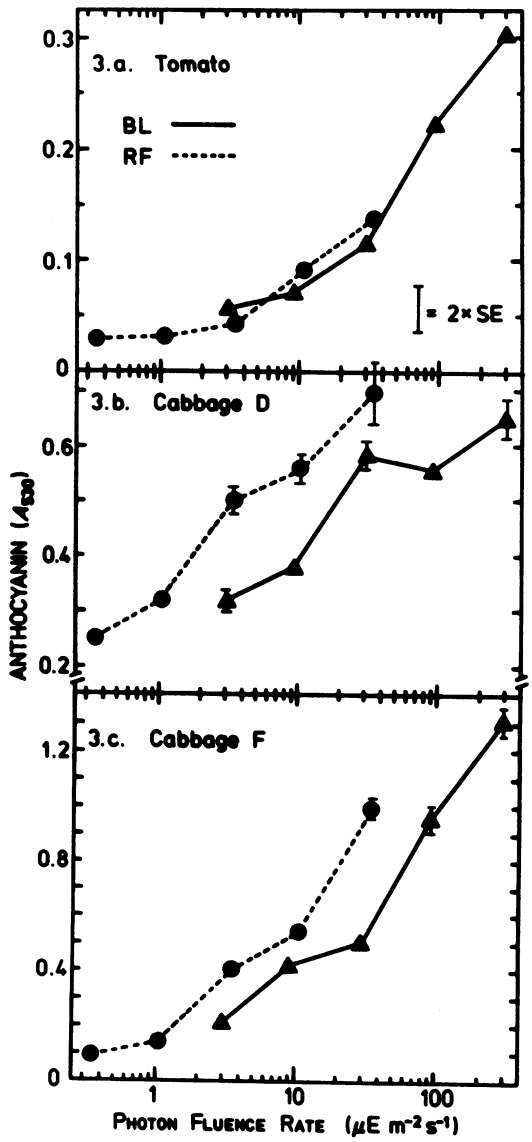

FIG. 3. Photon fluence rate dependence of anthocyanin production under BL and RF irradiations. See figure 2 for details.

the RF to BL quantum efficiency ratio of seven is quite close to the value of 8.87 for the σ_{RF}/σ_{BL} ratio. These results suggest two possible interpretations. Either cryptochrome is not involved in the photoregulation of the action of BL on anthocyanin production, or cryptochrome might be involved, but the state of phytochrome is the main limiting factor for the final expression of the response. With the type of experiments used in this study, it is not possible to decide definitively between these two alternatives.

In cabbage seedlings of strain F, when anthocyanin production is expressed as a function of k (Fig. 2c), BL is significantly more effective than RF. However, when anthocyanin production is expressed as a function of the photon fluence rate (Fig. 3c), RF is significantly more effective than BL. About 30 $\mu E\ m^{-2}\ s^{-1}$ of BL and 8 $\mu E\ m^{-2}\ s^{-1}$ of RF are required for the production of the same amount ($A_{530} = 0.5$) of anthocyanin. Thus, in terms of quantum efficiency, RF is about 3 to 4 times as effective as BL.

The situation in cabbage strain F seems to be intermediate between that in tomato and that in cabbage strain D. These results suggest that, in cabbage strain F, cryptochrome is involved in the photoregulation of the action of BL on anthocyanin production, and that the state of phytochrome might be more important than in tomato, but less important than in cabbage strain D as a limiting factor for the final expression of the response.

Comparative studies of the action of BL and RF on anthocyanin production, based on the application of the principle of equivalent light action, have some limitations. First, they provide a clear indication of cryptochrome involvement in the mediation of the action of BL only when, for equal values of the k and φ parameters of phytochrome, BL is significantly more effective than RF, as is the case for cabbage strain F and tomato. However, for situations like that in cabbage strain D, two equally reasonable interpretations can be suggested, and a different experimental approach must be used to distinguish between them. Second, they provide no information about the interaction between cryptochrome and phytochrome in the photoregulation of the response, again requiring that a different approach be used to determine the extent and temporal display of the interaction

between the two photoreceptors. Various studies suggest that the final expression of cryptochrome-mediated responses to BL might depend on the state of the phytochrome system (9, 11).

In conclusion, the results of our studies indicate clearly that cryptochrome is involved in the mediation of the action of BL on anthocyanin production in two of the three systems used. However, the results of the present study are not sufficient to determine which of the two alternative suggestions is valid for the situation observed in cabbage strain D, nor to make any reasonable suggestion about the extent and temporal display of the interaction between cryptochrome and phytochrome in the photoregulation of anthocyanin production in cabbage strain F and tomato. Further research is needed to clarify these two problems.

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