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Mode of coaction between blue/UV light and light absorbed by phytochrome in light-mediated anthocyanin formation in the milo (*Sorghum vulgare* Pers.) seedling

(blue light/UV action/coaction of photoreceptors/phytochrome action)

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ABSTRACT **Anthocyanin formation in milo (*Sorghum vulgare* Pers.) seedlings (coleoptile, mesocotyl, taproot) occurs only in white light and blue/UV light (BL/UV), while red light (RL) and far-RL are totally ineffective. However, after a BL/UV pretreatment, the participation of phytochrome can be demonstrated. With a short-wavelength light source [peak emission in longwave UV (UV-A)], the mode of coaction between BL/UV and light absorbed by phytochrome (RL) was studied with the following principal results. (*i*) As soon as the seedling becomes competent to respond to UV-A (with regard to anthocyanin formation), the involvement of phytochrome can be detected. (*ii*) A 5-min pulse of UV-A has a strong effect on the anthocyanin synthesis in the milo mesocotyl. This effect is fully reversible if a long-wavelength far-RL pulse (RG9 light) is given immediately after the UV-A light pulse. (*iii*) When seedlings treated with 5 min of UV-A and 5 min of RG9 light are kept in darkness for 3 hr and then transferred to RL, anthocyanin appears. (*iv*) In continuous UV-A treatment, anthocyanin accumulation starts after a lag phase of 3.5 hr (25°C). A RL pretreatment prior to the onset of UV-A treatment strongly increases anthocyanin accumulation in UV-A, though the lag phase is not affected. Moreover, a RL pretreatment does not affect the time course for escape from reversibility in UV-A. It is concluded from these data that BL/UV cannot mediate induction of anthocyanin synthesis in the absence of P_{fr}, the active form of phytochrome that absorbs maximally in the far-red. Rather, the action of BL/UV must be considered to establish responsiveness of the anthocyanin-producing mechanism to P_{fr}. P_{fr} operates in this system via two different channels. As the effector of the terminal response, it sets in motion the signal–response chain that eventually leads to the appearance of anthocyanin. This is a slow process with a lag phase of the order of 3.5 hr. The second function of P_{fr} is to determine the responsiveness to the effector P_{fr} in mediating anthocyanin synthesis. This is a very fast and highly sensitive phytochrome action that can be detected readily within 1 min. However, as long as the plant has not received BL/UV, the strong effect of RL on the effectiveness of P_{fr} remains cryptic. The effect of a RL pretreatment and the effect of a UV-A pretreatment on responsiveness towards P_{fr} (or, effectiveness of P_{fr}) were found to be totally independent of each other, even though it is the UV-A that permits operation of P_{fr}.**

Anthocyanin formation in the milo seedling (coleoptile, mesocotyl, taproot) occurs only in white light (WL) and blue/UV light (BL/UV), while red light (RL) and far-RL alone are totally ineffective (1, 2). The participation of phytochrome can be demonstrated following a BL/UV pretreatment (1, 2). Moreover, in addition to a BL/UV-A photoreceptor, cryptochrome, a specific UV-B photoreceptor is involved (3, 4); UV-A is longwave UV light between 320 and 400 nm; UV-B is shortwave UV light between 280 and 320 nm.

It was shown previously (1, 2) that any action of phytochrome on anthocyanin synthesis in milo depends on a pretreatment with BL/UV. However, the corresponding question of whether the expression of the BL/UV effect depends entirely on the far-RL-absorbing physiologically active form of phytochrome (P_{fr}) was not decided conclusively (2). In the present work we test the hypothesis (5) that the action of BL/UV establishes responsiveness towards P_{fr} rather than directly mediating induction of anthocyanin synthesis. In a number of plants a RL pretreatment—operating through phytochrome—strongly increases later responsiveness to P_{fr} (6). Thus, we also address the question of whether the effect of a RL pretreatment on responsiveness to P_{fr} can be detected in anthocyanin synthesis by milo seedlings, even though a BL/UV pretreatment is required before P_{fr} is able to mediate anthocyanin synthesis.

Finally, if the hypothesis that responsiveness to the "effector" P_{fr} is determined by ambient light (7) is accepted, one may expect that those light-mediated reactions that determine responsiveness to P_{fr} are fast compared to the time course of P_{fr}-mediated expression of the terminal photomorphogenetic response (anthocyanin synthesis in the present case). The data obtained in the present study support the concept (5) that P_{fr} is the only effector to operate on gene expression in photomorphogenesis; however, in many instances not only light absorbed by phytochrome but also BL/UV is required to establish or to increase and/or maintain responsiveness in a plant towards the effector P_{fr}.

MATERIALS AND METHODS

Caryopses of *Sorghum vulgare* Pers. cv. Weider (hybrid) were selected, and seedlings were grown at 25 ± 0.5°C as described (8). Standard light fields (7, 8) were used: WL, 36 $W \cdot m^{-2}$; RL, 6.8 $W \cdot m^{-2}$; BL, 7.0 $W \cdot m^{-2}$; UV-A light, 9.6 $W \cdot m^{-2}$ [a UV-A light source combined with a PG 218 Plexiglas filter to allow the action of a small amount of UV-B (for detailed information about spectral composition, see ref. 3); this UV-A was found to be particularly effective in anthocyanin induction in the milo seedling (Table 1)]; and long-wavelength far-RL (RG9 light), 10 $W \cdot m^{-2}$ ($\varphi_{RG9} < 0.01$). Anthocyanin was assayed at its long-wavelength peak absorbance at 520 nm as described (9). Our material contains only one red anthocyanin in unhydrolyzed extracts. Following in principle the procedure described by Stafford (10), we

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Abbreviations: D, dark; BL, blue light; RL, red light; UV-A, longwave UV light; RG9 light, long-wavelength far-RL obtained with an RG9-filter; WL, white light; P_{fr}, the far-RL-absorbing form of phytochrome; P_r, the RL-absorbing form of phytochrome; P_{tot}, total phytochrome; $\varphi_\lambda = [P_{fr}]_\lambda/[P_{tot}]$.

Table 1. Amounts of anthocyanin in the mesocotyl and the coleoptile of 72-hr-old milo seedlings

Light source	Amount of anthocyanin, A_{520}	
	Mesocotyl	Coleoptile
RL (6.8 W·m^{-2})	0.00	0.00
BL (7.0 W·m^{-2})	0.42 ± 0.02	0.27 ± 0.01
WL (36 W·m^{-2})	0.46 ± 0.01	0.31 ± 0.02
UV-A (quartz, 9.9 W·m^{-2})	0.72 ± 0.02	0.46 ± 0.01
UV-A (PG 218, 9.6 W·m^{-2})	0.70 ± 0.02	0.46 ± 0.01
UV-A (WG 305, 7.3 W·m^{-2})	0.66 ± 0.02	0.43 ± 0.02
UV-A (WG 345, 6.9 W·m^{-2})	0.45 ± 0.02	0.34 ± 0.01

Dark-grown seedlings 48 hr old were irradiated for 24 hr with the light sources and filters as indicated. The UV-A source was used in combination with different filters. Numbers of the WG cut-off filters designate the wavelength of 50% transmission. In all of the experiments, the UV-A source was used with a PG 218 Plexiglas filter to allow the action of a small amount of UV-B (3).

confirmed that this red anthocyanin is an acylated cyanidin glycoside. Under the extraction conditions used in the present experiments, the peak of absorbance of this anthocyanin is at 520 nm.

In vivo phytochrome measurements in the mesocotyl of milo were performed in E. Schäfer's laboratory as described (8) with a custom-built dual-wavelength spectrophotometer (Ratiospect).

Statistics. The data presented are mean values and are based on 30 (6 independent) replicates. Estimates of the SEMs are on the order of a few percent, depending on the complexity of the experimental treatments.

RESULTS

Phytochrome Photoconversion ($P_r \rightarrow P_{fr}$) in RL and UV-A. Time courses of photoconversion of the inactive RL-absorbing form of phytochrome, P_r, to P_{fr} were determined in the mesocotyl tissue of milo in RL (6.8 W·m^{-2}) and in UV-A (PG 218, 9.6 W·m^{-2}) (Fig. 1). With both light sources, the photoconversion was a first-order process. The photoequilibrium in UV-A was 0.74 compared to 0.8 in RL.

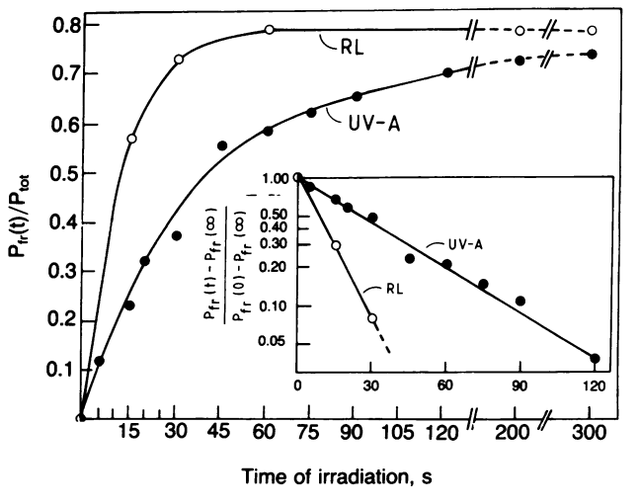

FIG. 1. Time courses of $P_r \rightarrow P_{fr}$ photoconversion in mesocotyl tissue in UV-A and in RL. Dark-grown seedlings 72 hr old were irradiated, and the P_{fr}/P_{tot} (total phytochrome) ratio was determined after different times of irradiation. (*Inset*) Semilogarithmic plot of the data. $P_{fr}(0)$, amount of P_{fr} at time = 0—i.e., before the onset of light; $P_{fr}(t)$, amount of P_{fr} after different times of irradiation; $P_{fr}(\infty)$, amount of P_{fr} after infinite irradiation.

Anthocyanin Accumulation in Continuous UV-A. The mesocotyl of the milo seedling does not produce red anthocyanin in complete darkness. As described (1, 2), even long-term RL or far-RL does not lead to any anthocyanin synthesis. However, UV-A caused a strong and rapid pigmentation (Fig. 2). Competence of the milo mesocotyl to respond to a WL or BL/UV treatment with anthocyanin synthesis appeared before 48 hr after sowing. However, the onset of inductive light (UV-A) was always 72 hr after sowing in the present experiments, since by this time point the mesocotyl had reached the highest state of competence of the three ages tested (Table 2). Under these circumstances, the lag phase before anthocyanin becomes detectable was 3.5 hr (Fig. 2 *Inset*).

Is There an Inductive Effect of UV-A on Anthocyanin Synthesis Independent of P_{fr}? Experiments with RL and far-RL pulses given after an inductive WL or BL/UV period of 3 hr have shown that the expression of the BL/UV effect is strongly modulated by P_{fr} (2). Moreover, in experiments with dichromatic irradiations—i.e., simultaneous irradiation with two kinds of light to modulate the level of P_{fr} against a constant background of BL/UV—it has been found that the effect of a BL/UV treatment is not affected by the presence or virtual absence of P_{fr} during this BL/UV treatment (2). However, the crucial question of whether an inductive effect of BL/UV on anthocyanin synthesis exists that is independent of phytochrome was not answered (2). The following experiments were designed to address this question.

Appearance of competence for UV-A and for phyto-

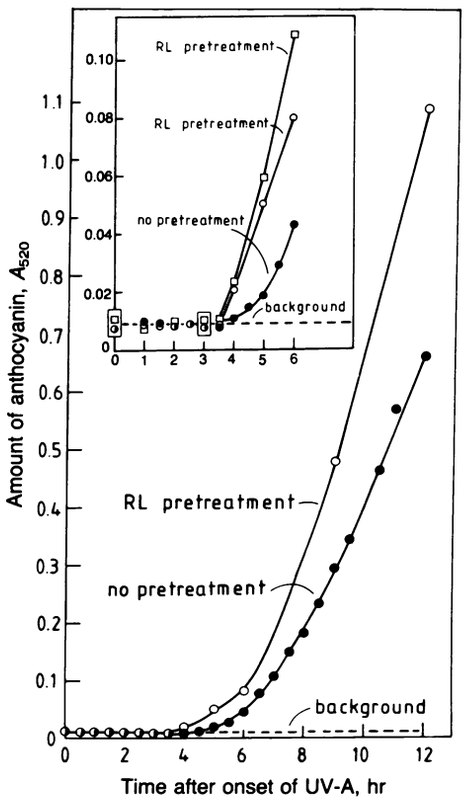

FIG. 2. Accumulation of anthocyanin in continuous UV-A treatment. ●, Seedlings were kept in darkness until 72 hr after sowing (zero time). ○ and □, Effect of 3- and 6-hr RL pretreatments, respectively, on the time course of accumulation of anthocyanin in continuous UV-A. Seedlings received a RL pretreatment from 69–72 hr (○) or 66–72 hr (□) after sowing. The accumulation of anthocyanin in UV-A was followed in the mesocotyl between 72 and 84 hr after sowing. (*Inset*) Detailed experiments to study the lag phase after RL pretreatments.

Table 2. Amount of anthocyanin in the mesocotyl of milo seedlings after 24-hr of UV-A (PG 218, 9.6 $W \cdot m^{-2}$)

Treatment*	Amount of anthocyanin, A_{520}
48 hr D/24 hr UV-A	0.70 ± 0.02
72 hr D/24 hr UV-A	2.41 ± 0.13
96 hr D/24 hr UV-A	1.71 ± 0.12

*The onset of light was 48, 72, or 96 hr after sowing. D, darkness.

chrome. In these experiments (onset of UV-A at the time of sowing), anthocyanin was extracted from the whole shoot because in continuous light the minute mesocotyl remains largely inside the testa. Since the primary leaf does not produce anthocyanin as it is inside the coleoptile, "shoot anthocyanin" is essentially "coleoptile anthocyanin." Even a 27-hr treatment with continuous UV-A did not cause any anthocyanin synthesis (Fig. 3). However, with 30 hr of UV-A, traces of anthocyanin could be detected at the time of assay (72 hr after sowing). Thus, the competence point for light lies between 27 and 30 hr after sowing. A difference between those shoots that received a RL pulse ($\varphi_{RL} = 0.8$) or a long-wavelength far-RL pulse ($\varphi_{RG9} < 0.01$) after termination of UV-A could be detected from the point of competence onwards. This means that the milo seedling responds to P_{fr} as soon as it responds to UV-A. There is no indication of a temporal separation of UV-A and P_{fr} actions.

Evidence that UV-A induces responsiveness to P_{fr}. Fig. 4 shows that the action of UV-A on the anthocyanin synthesis were inductive. Even 5 min of UV-A sufficed to induce responsiveness towards RL—i.e., towards P_{fr}. However a 5-min treatment with UV-A was ineffective—as far as anthocyanin synthesis is concerned—without P_{fr}. This is documented by the ineffectiveness of the UV-A treatment up to 10 min (with regard to the appearance of anthocyanin), provided that virtually all P_{fr} is returned to P_r at the end of the UV-A treatment by a saturating long-wavelength far-RL pulse ($\varphi_{RG9} < 0.01$). Clearly, 5 min of UV-A achieved something, namely responsiveness to P_{fr}, but 5 min of UV-A alone did not suffice to mediate anthocyanin synthesis.

On the other hand, UV-A induced responsiveness to P_{fr} so rapidly that, after 15 min of UV-A, the inductive effect was

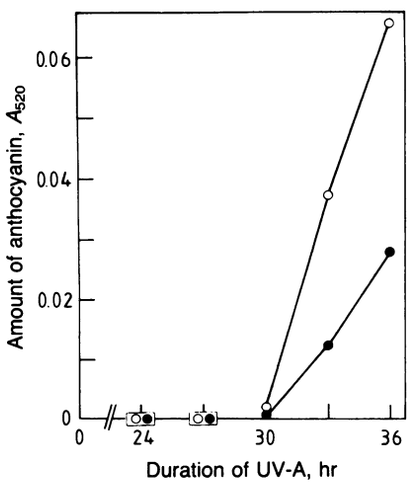

FIG. 3. Effect of UV-A treatment of different duration [onset at the time of sowing (zero time)] on anthocyanin accumulation in the shoot of the milo seedling. Immediately after the end of the UV-A treatment, the seedlings received either a 5-min RL pulse (○, φ_{RL} = 0.8) or a 5-min RG9-light pulse (●, $\varphi_{RG9} < 0.01$) to establish a maximum difference in P_{fr} content before the seedlings were placed in darkness until extraction for assay 72 hr after sowing. During the various dark periods, no loss of anthocyanin could be detected.

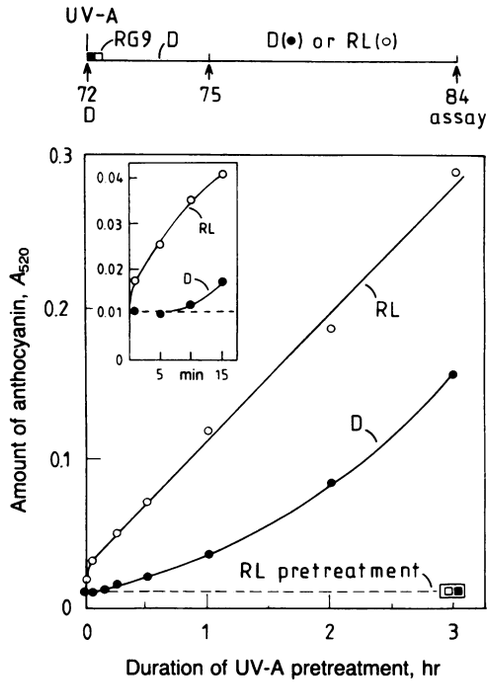

FIG. 4. (*Lower*) The effect of UV-A pretreatment on the accumulation of anthocyanin in the mesocotyl in subsequently given RL (○) or darkness (●). Dark-grown seedlings 72 hr old (i.e., at zero time) were irradiated with UV-A of different duration (abscissa). The UV-A treatment was terminated by a RG9-light pulse, which returns almost all P_{fr} to P_r. From 75–84 hr after sowing, seedlings were either kept in darkness (●) or in RL (○). For UV-A treatment of less than 3 hr, an appropriate dark interval separated the end of the [UV-A/RG9-light] treatment and the onset of RL. (*Upper*) A typical experimental protocol in which numbers refer to hours after sowing and D refers to darkness. (*Inset*) Enlargement of the results obtained during the first 15 min of light treatment (different set of experiments). □, RL pretreatment: RL given instead of UV-A; this value is identical with the control in complete darkness (■). This absorption is not due to anthocyanin.

no longer fully reversible by the terminating RG9-light pulse. Thus, in the presence of UV-A, P_{fr} can perform its initial action* within 15 min, even though it requires 3.5 hr before anthocyanin appears (see Fig. 2).

Is an Effect of RL on Responsiveness to P_{fr} (6) Detectable Besides the UV-A Effect on Responsiveness? In continuous UV-A treatment started 72 hr after sowing, anthocyanin accumulation commenced after a lag phase of 3.5 hr (25°C) (Fig. 2). A RL pretreatment prior to the onset of UV-A treatment strongly increased anthocyanin accumulation in UV-A even though the lag phase was not affected (Fig. 2 *Inset*). Moreover, a 3-hr RL pretreatment did not change the time point of escape from reversibility in UV-A by a terminating far-RL pulse (Fig. 5). Hence, a RL pretreatment does not affect the onset of the initial action* of P_{fr} in UV-A.

The data show that RL exerts a strong effect on responsiveness to P_{fr} (with regard to anthocyanin synthesis). However, this effect remains cryptic until UV-A establishes responsiveness to P_{fr}. It seems that the effect of the RL pretreatment (cryptic *per se*) is simply superimposed on the UV-A effect.

Can the Effect of a RL Pretreatment on Responsiveness to P_{fr} Be Separated Kinetically from the Action of P_{fr} in Mediating

*The term "initial action" designates the action of P_{fr} on some cell function that is no longer reversible upon the removal of P_{fr}. The onset of this initial action is defined by the escape from full reversibility (4).

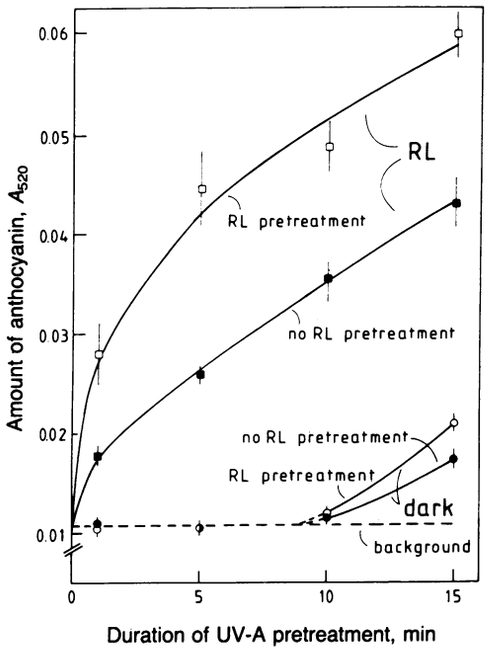

FIG. 5. The effect of a RL pretreatment on the escape from full reversibility in UV-A; 72-hr dark-grown seedlings (■, ●) and 69-hr dark/3-hr RL-grown seedlings (□, ○) were irradiated with UV-A for the time indicated in the abscissa. The UV-A treatment was terminated by a saturating RG9-light pulse. Until 75 hr after sowing, seedlings were kept in darkness. From 75–84 hr after sowing, the seedlings were either kept in darkness (○, ●) or in RL (□, ■). Anthocyanin extraction from the mesocotyl was performed at 84 hr after sowing.

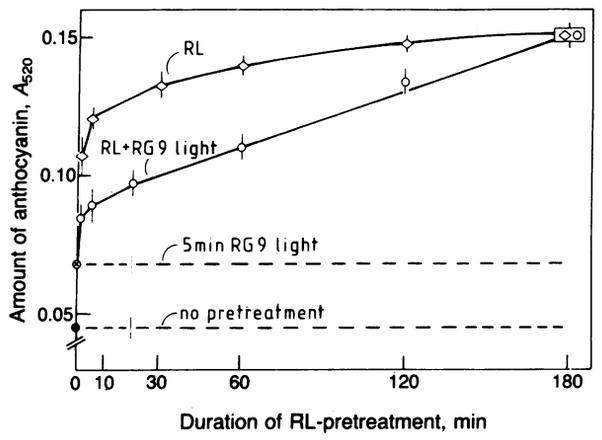

FIG. 6. The effect of a RL and far-RL pretreatment on the amount of anthocyanin extractable after a 6-hr UV-A treatment. At 69 hr after sowing (zero time), seedlings received one of the following treatments: a RL treatment (◇), a RL treatment followed by a subsequent 5-min RG9-light pulse (○), a 5-min RG9-light pulse alone (⊗), or no pretreatment (●). The seedlings were otherwise kept in darkness, and from 72–78 hr after sowing, they were irradiated with UV-A. Anthocyanin extraction from the mesocotyl was performed at 78 hr after sowing.

the Terminal Response? The RL effect on responsiveness to P_{fr} was extremely fast in the sense that even a 1-min RL pulse was no longer fully reversible (Fig. 6). Moreover, the extent of the reversible response decreased beyond 30 min, indicating as well rapid action of P_{fr}. The RL effect is extremely sensitive to P_{fr} because even a RG9-light pulse ($\varphi_{RG9} < 0.01$) exerted a substantial effect.

A comparison of the effectiveness of RL in Figs. 1 and 6 shows that an increase of the inductive effect of RL is observed beyond the point at which the photoequilibrium of phytochrome is established (1 min of RL). A similar phenomenon was observed previously in phytochrome-mediated anthocyanin synthesis in the mustard seedling (11). These facts indicate the rapid operation of a "high-irradiance reaction" of phytochrome (4).

DISCUSSION

The evidence obtained in the foregoing experiments indicates that UV-A cannot induce anthocyanin synthesis in the absence of P_{fr}. The action of UV-A must be considered to establish responsiveness to P_{fr} rather than mediating induction of anthocyanin. The same conclusion was drawn with regard to the action of BL on the longitudinal growth of the hypocotyls of sesame seedlings (7). RL absorbed by phytochrome contributes to the state of responsiveness as well, independently of UV-A. However, the RL effect on responsiveness remains cryptic until UV-A establishes responsiveness of the anthocyanin-producing mechanism to P_{fr}.

A mode of coaction between BL/UV and the light absorbed by phytochrome in light-mediated anthocyanin formation in the milo seedling is described by the scheme in Fig. 7. The major points are as follows.

(*i*) Phytochrome in the active form operates via two different channels. As the effector of the terminal response, it sets in motion the signal–response chain that eventually leads to the appearance of anthocyanin. This is a slow process with a lag phase of the order of 3.5 hr at 25°C (Fig. 2). A second function of phytochrome is to determine the

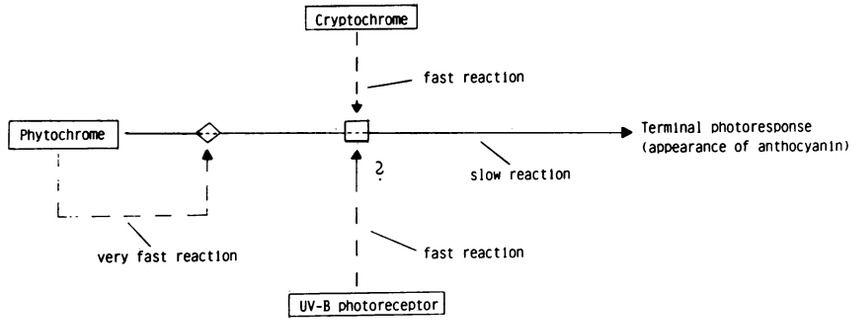

FIG. 7. Suggested mode of coaction between BL/UV and light absorbed by phytochrome in light-mediated anthocyanin formation in the milo seedling. The present scheme is based on present and previous (2, 3, 5–8) observations. →, Temporal sequence of events set in motion by the effector P_{fr}, leading to the terminal response; — → light-dependent reactions that determine the effectiveness of the effector P_{fr} (in other words, the responsiveness of the anthocyanin-producing mechanism towards P_{fr}). The point of action of UV-B, relative to the action of BL/UV-A, remains undecided at present (3).

responsiveness to the effector P_{fr} in mediating anthocyanin synthesis. This is a very fast and highly sensitive phytochrome action, which can be detected readily within 1 min (Fig. 6).

(*ii*) As long as the plant has not received BL/UV, the strong effect of RL on P_{fr} effectiveness remains cryptic (Figs. 2 and 5). It is BL/UV that opens the channel leading from the effector P_{fr} to the terminal response. The action of BL/UV is inductive: 1 min of UV-A leads to a significant effect (Fig. 4). However, the action of BL/UV is less fast and less sensitive compared to the action of RL on responsiveness towards P_{fr} (Figs. 4 *Inset* and 6).

(*iii*) In continuous UV-A, escape from full reversibility—i.e., the onset of the initial action of P_{fr}—can be detected within 15 min (Fig. 4). At present it remains an enigma why it requires more than 3 hr before anthocyanin eventually appears. As far as we know at present (5, 7, 8), responsiveness of a plant towards P_{fr} always depends on the quality and quantity of the ambient light. A higher plant measures light throughout the spectrum, and this information—obtained via phytochrome, cryptochrome, and a UV-B photoreceptor (4)—determines the efficiency of P_{fr} action, or in other words, the actual responsiveness towards P_{fr} during growth and development of a plant.

While it seems that the scheme in Fig. 7 represents the usual interdependence of BL/UV and light absorbed by phytochrome, it must be emphasized that, in most cases studied so far, a BL/UV treatment is not obligatory for a P_{fr} action to occur (5). Rather, BL/UV causes an intensification of P_{fr}-mediated processes, which occur even in RL alone, albeit at a low rate (8). The absolute requirement for a BL/UV treatment in the present case allows a complete experimental separation of the effect of BL/UV *per se* from that of phytochrome *per se*.

So far we have no knowledge about the "mechanism" of the fast reactions that determine the effectiveness of the effector P_{fr}. Irrespective of "mechanism," the coaction as sketched in Fig. 7 must be considered as highly economical because a single effector—namely, P_{fr}—suffices to bring about the molecular events leading to photomorphogenesis, and yet information about quality and quantity of light from the whole solar spectrum can contribute to the extent of the photomorphogenetic response.

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