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Phytochrome and Photosystem I Interaction in a High-Energy Photoresponse

(photosynthesis/photomorphogenesis/anthocyanin/turnip seedlings)

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ABSTRACT **At least two photoreactions can be demonstrated in plant developmental responses: the low-energy requiring phytochrome system and the high energy reaction. The action of these photoreactions on the formation of anthocyanin by turnip seedlings is discussed. The synthesis of small amounts of anthocyanin can be controlled solely by phytochrome, as evidenced by the red-far-red photoreversible effect of brief irradiations. Appreciable synthesis requires prolonged irradiations, the duration of irradiation being more important than intensity. The data presented suggest that the energy dependence of anthocyanin synthesis arises through photosynthesis. A mechanism for the interaction between photosynthesis and phytochrome is suggested. Under conditions of natural illumination of plants, the concentration of the species of phytochrome that absorbs far-red light may be lower than previously realized.**

Eukaryotic plant cells that are potentially photosynthetic all seem to contain the pigment phytochrome (1). The absorption of light by phytochrome can elicit changes in cellular electric potentials (2) and ion fluxes (3, 4), changes in cellular metabolism, and, ultimately, changes in growth and development (5). The characteristics of the phytochrome photoreaction through which these changes arise are partially understood. Phytochrome has absorption maxima near 664 and 724 nm (6, 7). Red light causes partial transformation of the 664-nm absorbing species, red-absorbing phytochrome (Pr) into the 724-nm absorbing species, far-red absorbing phytochrome (Pfr). The reverse transformation of Pfr to Pr by far-red radiation is more complete, and to some extent can occur spontaneously in darkness. Thus, responses that are regulated by phytochrome exhibit photoreversibility: responses potentiated by red light can be negated by far-red light.

The discovery of the phytochrome photoreaction led to the recognition of a second photoreaction that is dependent upon energies greater than those required for the phototransformations of phytochrome, and is designated the high-energy reaction (HER) (8–10, 5). HER responses typically have action maxima near 450 and 720 nm. Rarely, red action is evident. The most thoroughly studied HER response with a red action maximum is the synthesis of anthocyanin pigments by apples (11–14). It was concluded that this response arose through photosystem II of photosynthesis and phytochrome.

The photoreceptor(s) for HER responses is currently of principal concern. Recent models (15–17) for the origin of the HER are based upon phytochrome as the sole photoreceptor. Accordingly, HER are believed to arise through the maintenance of a low level of Pfr over a prolonged time. Indeed, the phytochrome and HER photoreactions appear closely linked, since photoresponses that exhibit evidence of a HER also exhibit phytochrome photoreversibility under appropriate conditions. The dependence of HER photoresponses on intensity remains more difficult to explain, and is the principal subject of this communication.

Originally, the involvement of photosynthesis in HER responses was suggested by Hendricks, Borthwick, and their associates (5, 13, 14). The participation of photosynthesis in responses with far-red action maxima grew to be doubted because of the location of the action maximum near 720 nm, the demonstration of HER responses in "dark-grown" seedlings, and adequate models to explain the responses on the basis of phytochrome.

We have undertaken a re-examination of the possible involvement of photosynthesis in a particular HER response, the synthesis of anthocyanins by turnip seedlings. This system has been extensively studied by Grill and Vince (20–25), and was selected by us because if photosynthesis were to be involved in far-red HER responses, it should be demonstrable in a system undergoing organization of the photosynthetic apparatus. Other HER responses (18, 16, 19) occur in fully green tissues, which have a greater potential for photosynthetic participation.

This report has three purposes. (*i*) The photoreactions controlling anthocyanin synthesis in turnip seedlings are characterized in greater detail. (*ii*) Data are presented that support the involvement of phytochrome and photosystem I of photosynthesis in anthocyanin synthesis. (*iii*) Suggestions are made as to how phytochrome and photosystem I may interact in HER responses.

MATERIALS AND METHODS

Seeds of Purple-Top, Which Globe Turnip (*Brassica rapa* L.) were purchased from the Burpee Co. Fine-White Mustard seeds (*Sinapis alba* L.) were purchased from Thompson and Morgan Ltd., Ipswich, Essex, England. The seeds, in lots of 100, were germinated on filter paper moistened with distilled water (25). The experimental light–dark schedules and treatments are described in the individual tables and figures.

Pigment Extractions and Measurements. Anthocyanin was extracted from seedlings in 0.01 molal HCl–aqueous 25% 1-propanol (see ref. 25 for details). The absorbance of the extraction solution at 535 nm was determined. Chlorophylls were extracted from seedlings by grinding in 80% acetone

Abbreviations: HER, high-energy reaction; Pr, red-absorbing phytochrome; Pfr, far-red absorbing phytochrome; Cl_2PhMe_2 Urea, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

under dim green light at 4°. The homogenate was centrifuged, and the absorbance of the supernatant was determined at 645 and 663 nm. Quantitative estimations of the chlorophyll content were obtained by use of the equations of Holden (26).

Light Sources. Where red and far-red appear, they refer to broad spectrum sources obtained through Plexiglas filters (25). At seedling level the intensities of the red and far-red sources were 0.14 and 4.5 mW/cm², respectively. The source used for action spectra and reciprocity determinations was obtained from (General Electric) incandescent narrow spot-lamps of 500 W filtered through 9 cm of flowing water. This source was used unfiltered or with interference filters with bandwidths less than 15 nm (Corion Instrument Corp., Waltham, Mass.).

RESULTS

The photoreactions

The action maximum for anthocyanin synthesis in turnip seedlings is located near 720 nm (Fig. 1), as has been reported (8, 24, 25). Fig. 1 reveals, however, that exposure times greater than 6 hr are required for the development of the action maximum when continuous radiation of about 1.0 mW/cm² is provided. The action maximum is evident after 12-hr exposure and is well developed by 24 hr, at which time a weak shoulder of red activity becomes apparent (Fig. 1).

The importance of intensity and duration of incandescent-lamp radiation on anthocyanin synthesis was studied. In the photoreactions controlling anthocyanin synthesis, duration appears somewhat more important than the intensity of the irradiation, since with equal energies [12 hr at 5000 ft-c or 24 hr at 2500 ft-c (1 foot-candle = 0.09 candela)] more anthocyanin is synthesized with longer exposure times (Fig. 2). This dependence on duration can be attributed in part to the induction phase (8, 23). Reciprocity still fails, however, during steady-state conditions after a 3-hr induction period (Table 1).

In the absence of prolonged irradiations, phytochrome alone can initiate anthocyanin synthesis in turnip seedlings: anthocyanin synthesis potentiated by a 5-min irradiation with red light is photoreversed by a 5-min irradiation with far-red (Table 2). The amount of anthocyanin synthesized in darkness after a single 5-min red irradiation is minimal. The promotion of anthocyanin synthesis by Pfr is dependent upon

TABLE 1. *Anthocyanin formation in turnip seedlings after indicated exposure and intensities of incandescent-lamp radiation*

Light intensity ft-c	Exposure (hr)	Absorbance at 535 nm*
Dark	0	0.055
600	3	0.095
2500	24	0.491
5000	12	0.175
600; 2500	3; 24	0.546
600; 5000	3; 12	0.193

Seedlings grown in darkness for 72 hr were harvested immediately after the irradiations.

* Values are the mean of 10 replicates, of 100 seedlings each.

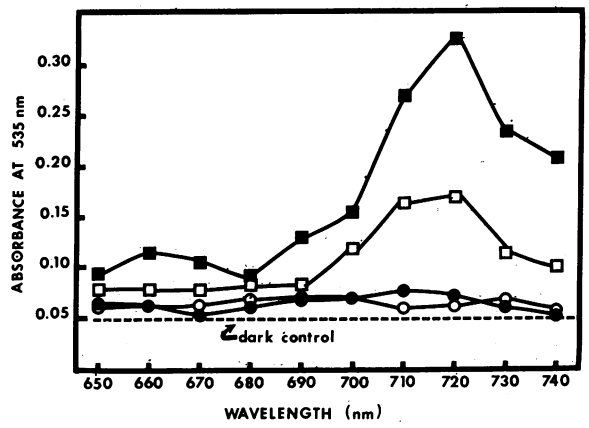

FIG. 1. Effect of exposure time and wavelength on anthocyanin formation in turnip. Dark-grown, 72-hr-old seedlings received radiation of 1.0 mW/cm², then were harvested immediately. Values are the mean of four replicates, of 100 seedlings each. ○——○, 3 hr; ●——●, 6 hr; □——□, 12 hr; ■——■, 24 hr.

seedling age, and is visible in 48-hr-old seedlings, but not in older seedlings. The Pfr promotion during a subsequent 24-hr dark period is enhanced considerably when a 5-min red irradiation is preceded by a 24-hr exposure to far-red light (Table 3). The promotion of turnip seedling anthocyanin

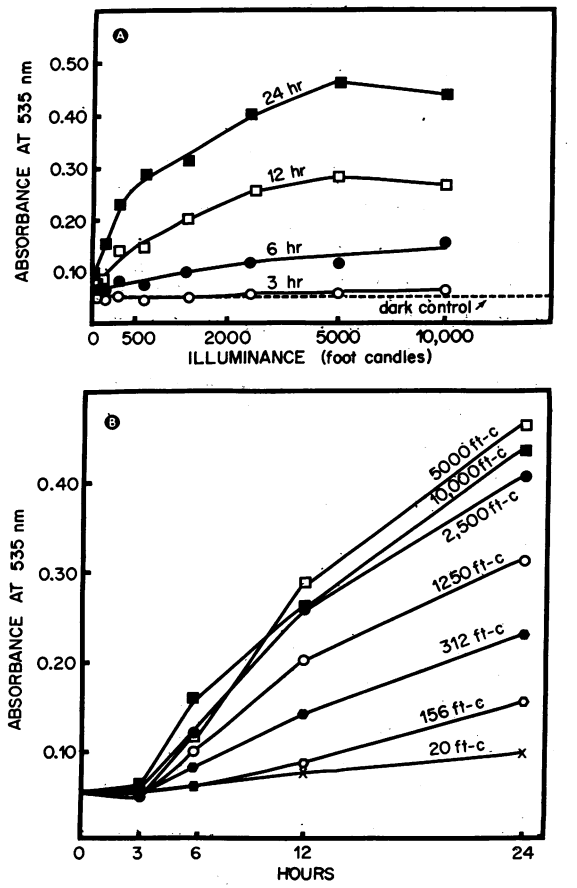

FIG. 2. Anthocyanin formation in turnip seedlings plotted as a function of incandescent lamp intensity (*A*) and exposure time (*B*). Seedlings grown in darkness for 72 hr were harvested immediately after the irradiations. Values are the mean of four replicates, of 100 seedlings each.

TABLE 2. *Formation of anthocyanin in response to brief irradiations*

Irradiation	Age (hr) at irradiation	
	48	72
	*Absorbance at 535 nm**	
None	0.049 ± (0.001)	0.064 ± (0.002)
5-min red	0.055 ± (0.002)	0.063 ± (0.002)
5-min red, 5-min far-red	0.050 ± (0.002)	0.060 ± (0.003)

Turnip seedings were grown in darkness for 48 or 72 hr and then harvested 24 hr after the irradiations.

* Values are the mean of 16 replicates, of 100 seedlings each, with standard errors expressed within parentheses.

formation by red light in the absence of a prolonged irradiation is in agreement with work on mustard seedlings by Lange *et al.* (27), who provided rigorous proof that phytochrome alone can mediate anthocyanin synthesis. The enhancement of the phytochrome promotion of anthocyanin formation by a previous prolonged irradiation supports earlier work on turnip seedlings by Grill and Vince (22).

Inhibitor studies

Cyclic and noncyclic photophosphorylation are activated preferentially by far-red and red light, respectively (28). Consequently, if cyclic photophosphorylatin were to represent a component of the synthesis of anthocyanin by turnip induced by far-red light, anthocyanin synthesis should be inhibited by inhibitors of cyclic, but not noncyclic, photophosphorylation. We have observed (25) that the effects of photosynthetic inhibitors on anthocyanin formation mimic their effects on cyclic, but not on noncyclic, photophosphorylation. The inhibitors affect mustard seedlings in a similar manner (Table 4).

We reported (25) that Pfr action and phytochrome photoreversibility were maintained in the presence of 0.1 mM dinitrophenol. Phytochrome control of anthocyanin synthesis is also maintained in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Cl_2PhMe_2 urea), *o*-phenanthroline, and antimycin A (Table 5). Thus, these inhibitors do not appear to be affecting the turnip HER response by destroying Pfr action and photoreversibility.

It can be argued that the inhibition of anthocyanin synthesis in the presence of dinitrophenol and antimycin arises through their inhibition of oxidative phosphorylation. We have tested the effects of a specific uncoupler of photophosphorylation, ammonium chloride (29), which inhibits turnip-seedling anthocyanin synthesis (Fig. 3).

TABLE 3. *Promotion of anthocyanin formation by red light in response to prolonged far-red radiation*

Treatment	Absorbance at 535 nm*	Red-light promotion
24-hr dark	0.051	
24-hr dark + 5-min red	0.057	0.006
24-hr far-red	0.377	
24-hr far-red + 5-min red	0.400	0.023

Turnip seedlings grown in darkness for 24 hr were treated as indicated, returned to darkness for 24 hr, and harvested at 72 hr.

* Values are the mean of 12 replicates, of 100 seedlings each.

Radiation at 720 nm increased the chlorophyll *a* content of turnip seedlings (25). Consequently, it could be predicted that if chlorophyll synthesis is inhibited, anthocyanin synthesis via the HER should also be inhibited. Levulinic acid acts as an effective inhibitor of chlorophyll synthesis in *Chorella* (30) and of anthocyanin synthesis in turnip seedlings (Fig. 4). Anthocyanin synthesis is restored upon removal of levulinic acid by a 30-min wash with distilled water (Fig. 4). In conjunction with the inhibition of anthocyanin synthesis, levulinic acid also inhibits chlorophyll *a* synthesis in turnip seedlings (Table 6). Levulinic acid did not lower endogenous levels of phytochrome assayable by spectrophotometry.

DISCUSSION

Our results indicate that phytochrome alone can mediate anthocyanin synthesis in turnip seedlings. Synthesis induced by brief irradiations sufficient to saturate phototransformations of phytochrome is minimal, however. Appreciable synthesis requires protracted radiation, during which both the duration and intensity are of consequence. The duration dependence probably arises in part through delays in the onset of photochemical activities of photosynthesis during greening. Oelze-Karow and Butler (31) recently reported that in bean leaves greened in far-red light, the onset of photosynthetic photochemical activities is prolonged. Photosystem I and *in vivo* cyclic photophosphylation developed before photosytem II. Photophosphorylation commenced after 12 hr of far-red radiation. These findings could explain in part the lag in development of the action maximum in Fig. 1. Plesnicar and Bendall (32) found that proplastids isolated from dark-grown barley leaves exhibit photosystem I activity immediately upon exposure to light, and that very high rates of cyclic photophosphorylation are detectible after 1 hr of illumination. The onset of photosystem I activity, whether in white or far-red light, is relatively rapid; its possible participation in photomorphogenic responses should not be neglected.

The results presented here and elsewhere (25) show that inhibitors of cyclic photophosphorylation inhibit the far-red HER response of turnip and mustard seedlings. Inhibitors of noncyclic photophosphorylation are not inhibitory. The inhibitors do not appear to be altering the HER response by

TABLE 4. *Anthocyanin formation in response to inhibitors*

Inhibitor	Species	
	Brassica rapa L.	*Sinapis alba* L.
	*Absorbance at 535 nm**	
None	0.258	0.428
Antimycin A, 10 μM	0.199	0.264
Dinitrophenol, 100 μM	0.148	0.223
Cl_2PhMe_2 urea, 0.2 μM	0.292	0.422
o-Phenanthroline, 100 μM	0.308	0.537

Seedlings, dark-grown for 48 hr, were supplied test solutions and then incubated in darkness for 24 hr. They then received 24 hr of far-red irradiation, followed by 24 hr of darkness, and were harvested at 96 hr.

* Values are the mean of no less than 15 replicates, with 100 seedlings each.

TABLE 5. *Effect of photosynthetic inhibitors on the red–far-red photoreversibility of anthocyanin formation in turnip seedlings*

Inhibitor*	None	Terminal irradiations: 5-min red	Terminal irradiations: 5-min red, 5-min far-red
	Absorbance at 535 nm†		
None (water control)	0.091 ± (0.002)	0.102 ± (0.003)	0.092 ± (0.003)
Cl_2PhMe_2 urea	0.093 ± (0.004)	0.103 ± (0.004)	0.094 ± (0.004)
o-Phenanthroline	0.114 ± (0.005)	0.138 ± (0.004)	0.116 ± (0.003)
Antimycin A	0.064 ± (0.003)	0.073 ± (0.004)	0.061 ± (0.001)

Inhibitors were supplied to 48-hr-old, dark-grown seedlings. After 40 hr of dark incubation, seedlings received an 8-hr far-red irradiation, terminated as indicated, and were then harvested after 24 hr of darkness.

* Concentrations used were: 0.2 μM Cl_2PhMe_2 urea, 100 μM *o*-phenanthroline, and 10 μM antimycin A.

† Each value is the mean of 14 replicates, of 100 seedlings each. Standard errors are expressed within parentheses.

acting directly on phytochrome. Photosynthetic pigments seem to be involved in the HER response of turnip, since when, chlorophyll synthesis is inhibited, the HER response is also inhibited. Thus, an aspect of photosystem I of photosynthesis, cyclic photophosphorylation, appears to be required for the response of turnip seedlings to prolonged far-red irradiations. We suggest that the intensity dependence of the turnip HER response reflects the intensity dependence of photosynthesis, whereas the duration dependence of this photoreaction arises through two components: delays in the onset of photosynthetic photochemical activities associated with greening and a requirement for the continued action and conservation of Pfr.

Although photosynthesis may contribute to HER responses, the mechanisms of its interaction with phytochrome remain

TABLE 6. *Effect of levulinic acid upon chlorophyll and anthocyanin content in turnip seedlings*

	Solutions*: Water	Solutions*: Levulinic acid (10.0 mM)
Anthocyanin content (absorbance at 535 nm†)	0.347 ± (0.013)	0.075 ± (0.003)
Chlorophyll content (μg/g fresh weight)		
Total	8.7 ± (0.7)	6.3 ± (0.7)
(*a*)	5.1 ± (0.4)	2.7 ± (0.2)
(*b*)	3.6 ± (0.4)	3.6 ± (0.4)

Seeds planted in indicated solutions were dark-grown, then received 24 hr of far-red irradiation. Seedlings were harvested after 24 hr of darkness at 96 hr.

* pH adjusted to 5.7 with KOH.

* Values are the mean for 11 or more replicates, of 100 seedlings each, with standard errors expressed within parentheses.

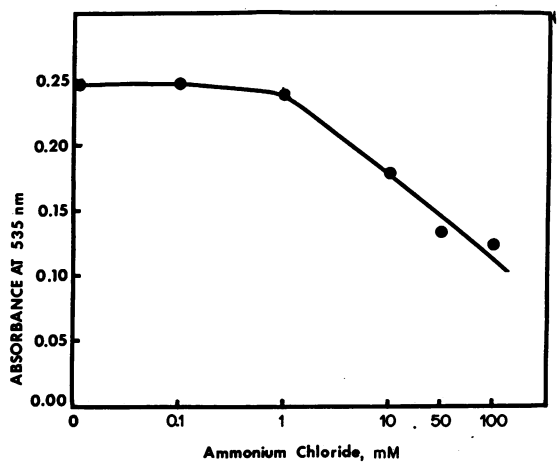

FIG. 3. Effects of ammonium chloride on far-red-induced anthocyanin formation. Turnip seedlings grown in darkness for 72 hr were irradiated for 24 hr, then harvested after 24 hr of additional darkness. Values are the mean of four replicates, of 100 seedlings each.

to be explained. Two mechanisms, which are not mutually exclusive, seem likely. The activation of cyclic photophosphorylation by far-red light could provide additional ATP, which is utilized in the development of HER responses. Data of Kandeler (33) and of Creasy *et al.* (34) show the enhancement of anthocyanin formation by incubation of red cabbage and strawberry tissues in exogenous ATP.

A second plausible mechanism for the interaction of photosynthesis with phytochrome is suggested by a recent report of Mumford and Jenner (35), who observed that the transformation of Pfr to Pr is promoted by various reductants. Reduced ferredoxin, a photosynthetic photoproduct, was the

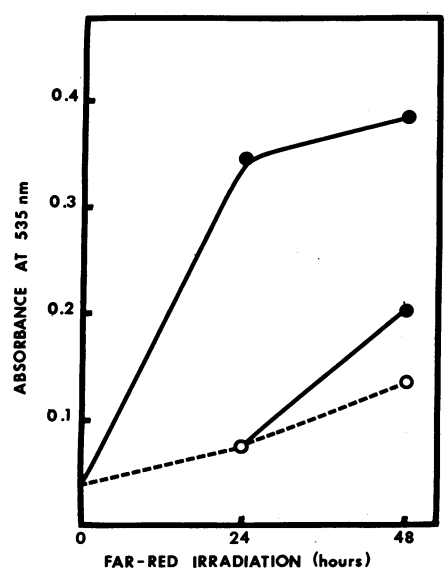

FIG. 4. The restoration of anthocyanin formation after inhibition by levulinic acid. Turnip seeds were planted in water (●——●) or 10.0 mM levulinic acid (O- - -O), incubated in darkness for 48 hr, irradiated, and then harvested after an additional 24 hr of darkness. Values are the mean of 11 replicates, of 100 seedlings each.

most effective reductant tested. It was not oxidized in the process, and it seemed to act catalytically in the transformation. Thus, *in vivo*, photosynthetic reductants could act to transform Pfr to Pr and thereby minimize Pfr destruction, and yet to participate in electron transport coupled to phosphorylation. Since radiation above 600 nm effects the production of reductants, the location of HER action maxima at wavelengths above 700 nm seems anomalous. Again the data of Mumford and Jenner (35) offer an explanation. Their absorption spectra of phytochrome reveal that the absorbance of Pfr at 725 nm after a saturating dose of red light is about 60% greater than after far-red exposure, even in the presence of reduced ferredoxin. Under steady-state conditions, Pfr absorbance and destruction would be minimized by wavelengths >700 nm, even though shorter wavelengths could lead to the production of reductants. Consequently, the action maxima for many HER responses appear near 720 nm.

These interpretations seem to apply to HER responses observed under conditions of natural illumination. We reported (19) that leaflets of the sensitive plant, *Mimosa pudica*, fold together (close) in darkness after a brief red irradiation. The leaflets remain unfolded (open) in darkness if the level of Pfr is lowered by a brief far-red irradiation. Thus, leaflet position in darkness can be regulated by phytochrome, as is evidenced by the red–far-red photoreversibility of this response. In sunlight (or even under artificial white-light sources) leaflets of *Mimosa* remain open during their daily photoperiods. Leaflets in sunlight close when placed in darkness. This would indicate that the Pfr/phytochrome ratio is sufficiently high so as to induce leaflet closure, but the fact remains that the leaflets are open in sunlight. To explain this anomoly we suggest that the *in vivo* level of Pfr in sunlight is lower than previously realized and that mechanisms proposed here may be operative; namely, the production of reductants through photosynthesis catalyzes the transformation of Pfr to Pr in sunlight and permits the leaflets to remain open. In darkness, cellular reducing potentials are lowered in the absence of photosynthesis and allow for sufficiently high Pfr/phytochrome ratios to account for the observed leaflet closure. Evolutionarily, the occurrence and restriction of phytochrome to cells with potential photosynthetic capacity possibly reflects the interrelations in nature between the photosystems of photosynthesis and phytochrome.

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