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Contributions of Photosynthesis and Phytochrome to the Formation of Anthocyanin in Turnip Seedlings

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ABSTRACT

Turnip seedlings (*Brassica rapa* L.) irradiated for 24 hours with radiation at 720 nanometers synthesize chlorophyll *a* and anthocyanin. Antimycin A and 2,4-dinitrophenol, which are known to reduce cyclic photophosphorylation, also reduce anthocyanin synthesis. Noncyclic photophosphorylation is inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea and *o*-phenanthroline. These compounds promote cyclic photophosphorylation and anthocyanin synthesis. On the basis of these findings it is suggested that the photomorphogenic response of anthocyanin synthesis in turnip seedlings arises in part through photosynthetic activity.

Phytochrome involvement in turnip seedling photomorphogenesis is evidenced by the photoreversibility of anthocyanin synthesis in response to 5-minute irradiations with red or far red light. The inhibition of anthocyanin synthesis by 2,4-dinitrophenol does not arise from a destruction of phytochrome photoreversibility.

It is suggested that plant photomorphogenic responses to prolonged far red irradiations arise through the photochemical activation of at least two pigment systems; namely, the photosynthetic pigments and phytochrome.

The role of phytochrome in plant photomorphogenesis is summarized in several review articles (18–20, 24–26). The salient features of this system include the existence of phytochrome in two principal forms Pr and Pfr with absorption maxima located near 660 and 730 nm respectively. Responses mediated by phytochrome and potentiated by red radiation are photoreversible by far red radiation. An irradiation as low as 0.02 mw/cm^2 at 660 nm can induce a phytochrome-controlled response (18). The Bunsen-Roscoe reciprocity law applies to the photoreversibility of phytochrome-controlled responses.

Photomorphogenic responses which exhibit phytochrome action are often modified by prolonged irradiations with high intensity far-red, red, or blue light (18). HER[1] is used to designate those photoreactions in which the energy levels required for a maximum response are above those required for the photochemical transformations between the Pr and Pfr forms of phytochrome. HER responses are as diversified as are phytochrome-controlled responses and include flowering (6, 21) nyctinastic leaf movements (12), hypocotyl lengthening and hook opening (18), and pigment synthesis (22).

Most current interpretations of far red HER responses are based upon phytochrome as the photoreceptor. A model to account for the role of phytochrome in far red HER responses was proposed by Hartmann (17). His model, while satisfactory in many respects, failed to explain adequately the intensity dependence of HER responses. Moreover, it does not account for some observations that far red HER responses can arise when the ratio of Pfr/P approached its maximum value of 0.80 (6, 12). These difficulties have been accommodated in a model proposed by Borthwick *et al.* (6). Still another model has been proposed by Smith (26) in which he points out that his own model is completely speculative and is not supported by any direct evidence.

The possible contribution of photosynthesis to HER responses was considered frequently by Siegelman and Hendricks (22, 23); Downs *et al.* (9, 10); Evans *et al.* (11). They observed the inhibition of a red HER response by DCMU (9, 10), but found this inhibitor of electron transport in photosynthesis to be ineffective for far red HER responses (11). The involvement of photosynthesis in energy-dependent photomorphogenesis was reported by Satter and Wetherell (28). The possible contributions of photosystems I and II of photosynthesis to HER responses were suggested by Downs (9) and Downs *et al.* (10). Bertsch and Mohr (4), however, pointed out that their own experiments excluded all possibilities of the photosynthetic apparatus being involved in the far red HER associated with anthocyanin synthesis in mustard seedlings. Presently, plant photomorphogenic responses are considered independently from photosynthesis even though prolonged irradiations are used (20).

In studying photomorphogenic responses the following points need be emphasized: cyclic photophosphorylation is activated maximally by far red radiation whereas noncyclic photophosphorylation is primarily a red light system. The effect of specific uncouplers and inhibitors of electron transport on ATP formation by noncyclic and cyclic photophosphorylation was reported by Arnon *et al.* (1). We have determined the effect of these same inhibitors on the far red-induced HER responses of anthocyanin synthesis in turnip seedlings.

MATERIALS AND METHODS

Seeds of Purple-Top White Globe Turnip (*Brassica rapa* L.) were purchased from the Burpee Company. Seeds in lots of 100 (lots of 500 for chlorophyll experiments) were germinated in darkness within uncovered Petri dishes on single 9-cm discs of Whatman No. 3 filter paper moistened with 7 ml of distilled water. Seedlings were dark grown at room temperature. Forty eight hours after planting, 10 ml of the appropriate test solution or water were added to each dish. After a subsequent 24 hr in darkness, seedlings were irradiated for 24 hr. During the irradiation, dishes were covered with dishes of equal diam-

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[1] Abbreviation: HER: high energy reaction.

eter to prevent desiccation and yet provide adequate aeration. The seedlings were removed to darkness for an additional 24 hr after which they were harvested. This schedule is summarized in Figure 1. Any deviations from this schedule are described in the individual experiments.

Chlorophyll Extraction and Measurement. Harvested lots of 500 seedlings were weighed and ground in 90% aqueous acetone at 4 C. The homogenate was vacuum filtered through Whatman No. 1 filter paper. Acetone was removed from the filtrate using a flash evaporator. The chlorophyll was then partitioned from the aqueous suspension into petroleum ether. The volume of this solution was reduced *in vacuo* and the pigments were chromatographed on a column of powdered sucrose at 4 C using 0.5% 1-propanol in petroleum ether as the solvent. Upon resolution, the chlorophyll *a* band was removed and eluted with fresh anhydrous diethyl ether. The above operations were carried out in darkness or under dim green safelight. The sucrose was removed by repeated cycles of centrifugation and washing, and the remaining solution of chlorophyll in ether was air-dried to a 3-ml volume. The absorption spectrum of the chlorophyll in ether was obtained with a Cary recording spectrophotometer Model 14R. Quantitative estimations of chlorophyll *a* content were determined by the method of Smith and Benitez (27) whereby:

$$C_a\ (g/1) = 0.0101\ D_{662} - 0.00101\ D_{644}$$

Anthocyanin Extraction and Measurement. Harvested lots of 100 seedlings were placed in tubes containing 15 ml of 0.01 molal HCl in aqueous 25% 1-propanol and held for 24 hr at 4 C. The tubes were then placed in a boiling water bath for 5 min. After boiling, the samples were filtered through glass wool which was washed with additional extraction solution until each sample equalled 25 ml. The absorbance at 535 nm was determined for each sample with a Coleman Spectrophotometer Model 6/20.

Light Sources. Light sources were constructed in this laboratory. Far red illumination was provided by six General Electric incandescent 150 w projector flood lamps filtered through 8 cm of water and a far red Plexiglas filter No. FRF-700 3 mm thick (West Lake Plastics, Lenni Mills, Pa.). All light intensities were measured with a YSI-Kettering Model 65 radiometer. At seedling level the intensity of the far red source was 4.5 mw/cm². The red source consisted of four General Electric F48PG17 cool white fluorescent lamps filtered through 3 mm of Rohm and Haas 2444 red Plexiglas. At seedling level the intensity was 0.14 mw/cm². The source used for action spectrum determinations was obtained from General Electric incandescent 500 w narrow spotlamps filtered through 9 cm of flowing water and interference filters with bandwidths less than 15 nm (Corion Instrument Corporation, Waltham, Mass.). At seedling level the intensity was 1.0 mw/cm².

RESULTS

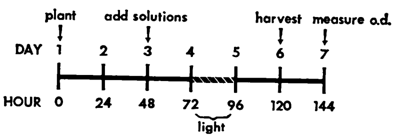

FIG. 1. General experimental schedule.

Table I. *Chlorophyll a Content of 5-Day-Old Turnip Seedlings*
Values are expressed as $\mu g \times 10^2$/g fresh wt.

Irradiation	Dark	Wavelength		
		680	700	720
hr			*nm*[1]	
0	3			
2		42	32	15
24		2400	1600	160

[1] Indicated wavelengths at 1.0 mw/cm².

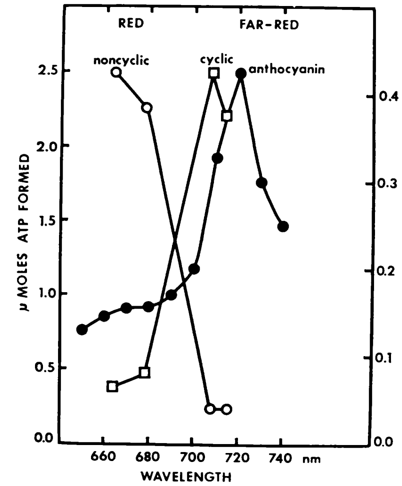

FIG. 2. Effect of red and far red spectral regions on anthocyanin synthesis by turnip seedlings and on ATP production by cyclic and noncyclic photophosphorylation. ATP values are from Arnon *et al.* (1).

Far red HER responses are as commonly associated with etiolated tissues as they are with green tissues, a fact which has provided indirect evidence for the lack of involvement of photosynthesis in these responses. It should be emphasized, however, that in order to elicit a HER response in etiolated as well as in green tissues, high energy irradiations must be provided. It is for this reason that we first measured the chlorophyll *a* content of turnip seedlings following actinic irradiations. Table I shows that the chlorophyll *a* content increases over the dark control value by a factor of five after 2 hr of 720 nm radiation and more than 50 times over the dark control value after 24 hr. Thus, in the process of inducing a far red HER response in turnip, chlorophyll *a* is synthesized and provides for potential photosynthetic activity.

Results obtained by Arnon *et al.* (1) reveal the differential effect of red and far red monochromatic light on noncyclic and cyclic photophosphorylation. Noncyclic photophosphorylation is a red light activated reaction, whereas cyclic photophosphorylation is activated most effectively by wavelengths > 700 nm. In this respect the far red light HER associated with anthocyanin synthesis in turnip seedlings resembles cyclic photophosphorylation. The similarities in the spectral requirements of these two photoreactions are shown in Figure 2. These results and the chlorophyll *a* data of Table I encouraged us to test inhibitors of cyclic and noncyclic photophosphorylations on the far red light-induced HER of turnip. The results are presented in Figures 3 through 6. Antimycin A and DNP are effective inhibitors of cyclic photophosphorylation, whereas the same concentrations of these compounds can cause a slight promotion of the noncyclic process. It can be seen in Figures 3 and 4 that concentrations of Antimycin A and DNP which reduce cyclic photophosphorylation also reduce the HER response of anthocyanin synthesis in turnip seedlings.

The effects of DCMU and *o*-phenanthroline on anthocyanin

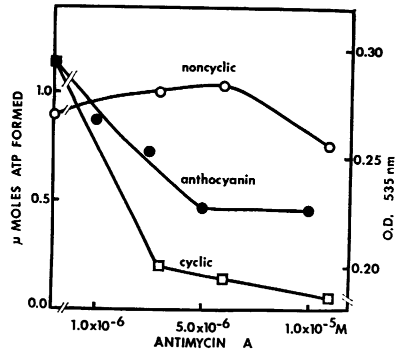

FIG. 3. Effect of Antimycin A concentrations on anthocyanin synthesis by turnip seedlings and on ATP production by cyclic and noncyclic photophosphorylation. Turnip seedlings were irradiated with 24 hr far red. ATP values are from Arnon *et al.* (1).

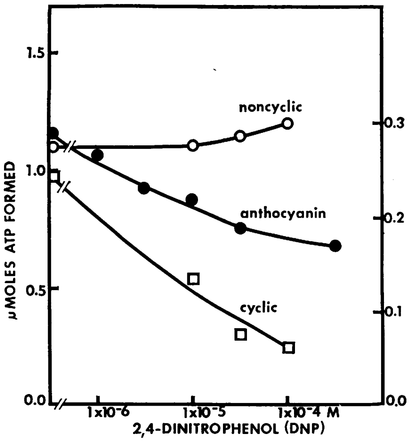

FIG. 4. Effect of DNP concentrations on anthocyanin synthesis by turnip seedlings and on ATP production by cyclic and noncyclic photophosphorylation. Turnip seedlings were irradiated with 24 hr far red. ATP values are from Arnon *et al.* (1).

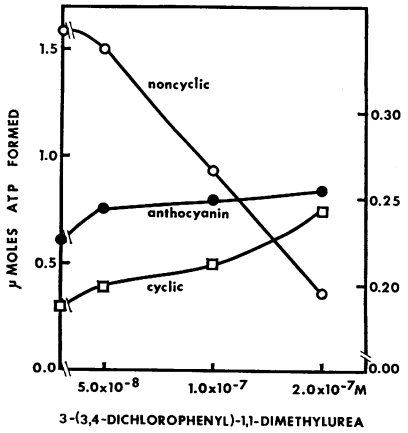

FIG. 5. Effect of DCMU concentrations on anthocyanin synthesis by turnip seedlings and on ATP production by cyclic and noncyclic photophosphorylation. Turnip seedlings were irradiated with 24 hr far red. ATP values are from Arnon *et al.* (1).

synthesis and cyclic and noncyclic photophosphorylations are shown in Figures 5 and 6. Again, the effect of these compounds on anthocyanin synthesis resembles their effect on cyclic rather than noncyclic photophosphorylation. Thus, with each of the compounds tested, the effect on the HER response mimics the effect on cyclic but not noncyclic photophosphorylation.

We have obtained preliminary but similar results with these same inhibitors on another HER response; namely, anthocyanin synthesis in fine white mustard (*Sinapis alba* L.). Also, we have observed that the recently reported inhibitor of chlorophyll synthesis (3), levulinic acid, also inhibits the HER response of anthocyanin synthesis in turnip cotyledons. We interpret this observation as an indication of the importance of photosynthetic pigments in HER responses.

The results above suggest a mechanism whereby pigments of the photosynthetic system can contribute to HER responses in etiolated seedlings undergoing simultaneous light-induced greening and anthocyanin synthesis. However, the photomorphogenic pigment, phytochrome, is also involved in the synthesis of anthocyanin as evidenced by the photoreversible nature of the response. Turnip seedlings which have received a 24 hr far red irradiation terminated with 5 min red light synthesize more anthocyanin during the next 24 hr in darkness than do those in which the terminal irradiation is far red light

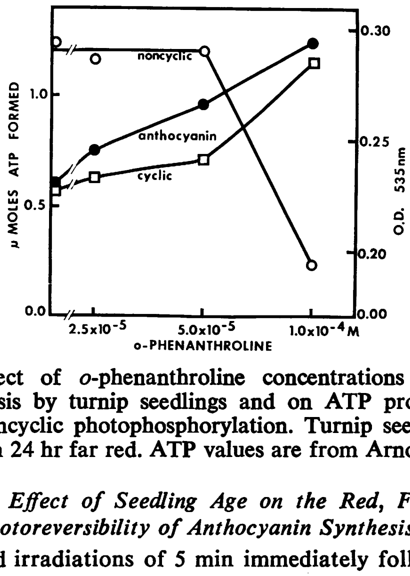

FIG. 6. Effect of *o*-phenanthroline concentrations on anthocyanin synthesis by turnip seedlings and on ATP production by cyclic and noncyclic photophosphorylation. Turnip seedlings were irradiated with 24 hr far red. ATP values are from Arnon *et al.* (1).

Table II. *Effect of Seedling Age on the Red, Far Red Photoreversibility of Anthocyanin Synthesis*

Red, far red irradiations of 5 min immediately followed a 24-hr far red irradiation. Values represent mean for 100 seedlings from four experiments with six replicates each.

Seedling Age	Mean *A* at 535 nm		
	Control	Red	Red, far red
hr			
48	0.270	0.335	0.283
72	0.468	0.477	0.483
96	0.271	0.269	0.261

TABLE III. *Effect of DNP on the Red, Far Red Photoreversibility of Anthocyanin Synthesis*

Red, far red irradiations of 5 min were given to 66-hr-old seedlings, 6 hr prior to a 24-hr far red irradiation. Values are the mean for 100 seedlings from two experiments with four replicates each.

Treatment	Mean *A* at 535 nm		
	Control	Red	Red, far red
H_2O	0.289	0.244	0.297
DNP (0.1 mM)	0.139	0.119	0.151

(Table II). This promotion of anthocyanin synthesis in darkness by Pfr induced by red light is most readily observed in younger seedlings. In contrast, Pfr can also inhibit anthocyanin synthesis in seedlings irradiated with red light 6 hr prior to a 24 hr far red irradiation (Table III). The inhibition potentiated by red light is photoreversible with far red light. As seen in Figure 4, 0.1 mM DNP inhibits anthocyanin synthesis, but as evidenced in Table III this inhibition does not appear to arise through a destruction of phyotochrome photoreversibility.

DISCUSSION

The photoreceptor pigments responsible for the red and far red HER responses of plant photomorphogenesis have been a subject of much recent speculation. Several models have been proposed to explain HER responses on the basis of phytochrome as the sole photoreceptor pigment.

In this paper the possible contribution of photosynthesis to a particular far red light-induced HER response, the photocontrol of anthocyanin synthesis by turnip seedlings, has been examined. The results show that chlorophyll *a* is synthesized when seedlings receive a prolonged irradiation of far red light at 720 nm. This increase in chlorophyll *a* affords the possibility of far red-induced photosynthesis.

Compounds which are known to affect photosynthetic electron transport were found to alter the HER response of turnip in a manner analogous to their effect on cyclic but not noncyclic electron transport. Thus, cyclic photophosphorylation seems to represent one aspect of the photochemical activity involved in the far red HER of turnip seedlings. Recently, Arntzen *et al.* (2) found that photochemical activity in greening *Zea mays* proceeds in a step-wise manner with cyclic photophosphorylation being detected after only 1-hr illumination of dark-grown seedlings. Activity after irradiations of less than 1 hr were not tested. NADP reduction could not be detected before 5 hr. Thus, the potential exists for phosphorylations via a cyclic pathway shortly after the onset of irradiation of dark grown seedlings.

Since it is possible that far red HER responses can arise in part from photosynthesis occurring during the greening process, it is of interest to consider far red HER responses of plants possessing their full complement of photosynthetic pigments. Previously, we observed that the HER associated with far red light-induced leaflet opening in fully green *Mimosa pudica* operates independent of the ratio of Pfr/P_{total} (12). While the Pfr/P_{total} ratio is important in controlling the dark reactions of photomorphogenesis in plants, the importance of the ratio during a prolonged high energy irradiation is less apparent.

The far red HER of anthocyanin synthesis in turnip seedlings seems to represent a composite response arising from at least two reactions: photosynthetic activity and the Pfr/P_{total} ratio in darkness. Creasy *et al.* (7, 8) have emphasized the importance of carbohydrate metabolism and photosynthesis in anthocyanin production by strawberry leaf disks. If indeed other far red HER arise by dual photochemical activities as we suspect they do, it would be of great interest to determine the pathways of CO_2 fixation in plants which exhibit far red HER responses. In contrast to plants with the reductive pentose phosphate cycle of CO_2 fixation, available data indicate (5) that plants which fix CO_2 via the C_4 dicarboxylic acid pathway are characterized in part by: higher levels of P_{700}, chlorophyll *a*, and higher rates of cyclic photophosphorylation associated with photosystem I. Moreover, photosynthesis in C_4 plants does not saturate at the usual intensities of 2000 to 3000 ft-c, but instead intensities approaching full sunlight (8000–10 000 ft-c) are required for saturation. Incandescent lamp radiation at 5000 ft-c does not appear to saturate the HER response of anthocyanin synthesis in turnip seedlings.

Our results on the contribution of phytochrome to anthocyanin synthesis confirm those of Grill and Vince (13–15) who have carried out extensive studies on the photocontrol of anthocyanin synthesis in turnip seedlings. They too have concluded that two photoreactions are involved (16). The results presented here attempt to identify these photoreactions.

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