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Relationships Between Phytochrome State and Photosensitive Growth of Avena Coleoptile Segments[1]

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Summary. Using various photostationary state light sources to obtain reproducible phytochrome conversion of from 5 to 88 % P_{FR}, assayed by 2 wavelength in vivo spectrophotometry, relationships between initial percent P_{FR} and elongation of apical *Avena* coleoptile segments over the succeeding 20 hours in darkness were studied. With material grown in total darkness, all P_{FR} levels promote elongation, and maximal promotion requires roughly 50 % P_{FR}. The promotion caused by an initial 5 minute red (88 % P_{FR}) treatment at hour 0 is partially reversible at hour 5 by sources forming less than 48 % P_{FR}, but totally irreversible at hour 8, though less than 50 % of the growth has been accomplished by this time. Direct photometric assays at hour 5 indicate a phytochrome state of roughly 45 % P_{FR}, consistent with the reversal data. At hour 8, however, 11 to 22 % of the phytochrome still assays as P_{FR}, an inconsistency suggesting simply that the elongation process has proceeded beyond photochemical control. Thus, in contrast with results previously reported for *Pisum* and *Phaseolus*, there is no contradiction between photometric and physiological assays of phytochrome state in *Avena* coleoptile segments.

Attempts to expand this study by using segments from seedlings pretreated with red light showed that such pretreatment as little as 1 to 2 hours before drastically reduces subsequent elongation and photoresponse on the medium employed. This decline in growth potential can be halted at any time before its completion by either excision of the segment or far-red treatment of the intact seedling.

It is generally accepted that the reversible photoreceptor, phytochrome, mediates the control of plant growth and development by low intensity red and far-red light. This pigment may be assayed in vivo by photometric techniques, thereby permitting the study of relationships between phytochrome changes and developmental events with a minimum disruption of the responding system. The evidence accumulated thus far indicates that such relationships are by no means clear and simple.

Phytochrome has been detected in young intact *Pisum* roots which do not respond to red and far-red light, while none could be found in the photosensitive, cultured root tissues (7, 8). The kinetics of disappearance of the far-red absorbing form (P_{FR}) in tissues of etiolated seedlings appear to bear no relationship to the photoperiodic class of the mature plant (11). No phytochrome could be detected in etiolated *Lemna perpusilla*, a highly sensitive photoperiodic plant (9). Even more puzzling are the results with growth responses of tissues pretreated with red light. Under conditions in which no P_{FR} could be detected by photometric assay, *Pisum* stem segments isolated from red pretreated plants respond to various light qualities as though approximately 20 % of the phytochrome were in the P_{FR} state (10). Again with P_{FR} photometrically undetectable, essentially complete far-red reversal of the growth response of *Phaseolus* hypocotyl hooks was obtained (6).

The effects of various light qualities on phytochrome conversion and growth in *Avena* coleoptile segments (12) have now been investigated with results in some ways unlike any yet reported.

Materials and Methods

Avena Coleoptile Segment Elongation. Seeds of *Avena sativa* L. var. Clintland were obtained from Southern States Cooperative, Baltimore, Maryland. Fifty ml (approx 28 g) of unhusked seeds were soaked for 4 hours in a tenth strength solution of Hutner's medium (10) and then scattered evenly over the surface of vermiculite in polyethylene basins. Each basin (12 x 8 x 4 inches) contained approximately 125 to 130 g coarse vermiculite wetted with 500 ml tenth strength Hutner's. The basins were

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covered with vented lids which reduced evaporation without seriously restricting gas exchange. Soaking of the seeds and planting were carried out in total darkness or under a dim green safe-light. The seedlings were allowed to develop at 26 to 27° in darkness for 3 days, after which those coleoptiles 10 to 20 mm long were selected. A single 6.00-mm apical segment, including the primary leaf, was cut from each coleoptile. The segments were randomized in glass-redistilled water and divided into lots of 10. Each lot was placed in a 50-ml beaker containing 3.0 ml of 0.02 M KH_2PO_4-Na_2HPO_4 buffer, pH 6.2, plus 1.5 % sucrose. Light treatments were initiated immediately after transfer of the segments to the buffered sucrose medium and the segments were incubated in darkness for 18 to 20 hours at 26 to 27°. The lengths were then measured to the nearest 0.5 mm with the aid of a stereomicroscope.

Phytochrome Assays. Phytochrome assays were conducted with a 2-filter difference spectrophotometer (Ratiospect model R-2, Agricultural Specialties Company, Beltsville, Maryland). This instrument measures the OD difference, or Δ OD, between 2 arbitrary wavelengths ($OD\lambda_2$-$OD\lambda_1$). In these experiments, λ_2 = 660 mμ and λ_1 = 730 mμ were established with interference and appropriate accessory glass color filters. Actinic red and far-red light for driving the pigment to one form or the other was obtained from a 500-w incandescent spotlight approximately 25 cm above the sample and separated from it by at least 5 cm of water. For red illumination, a 650 mμ interference filter with an accessory Corning CS 2-62 red filter was interposed. For far-red, a 3-mm thickness of the black Plexiglas described above was interposed. In each assay, 30 to 50 apical coleoptile segments (5.8 mm long), minus the enclosed primary leaf, were presented in a cuvette with an inside diameter of 3 mm. This method has been described in greater detail elsewhere (3, 4, 10, 11).

Studies on in vitro phytochrome preparations (5) indicate that even the most efficient red sources leave approximately 20 % of the red-absorbing form unconverted at saturation. Since there is presently no certain method for computing the degree of this incomplete conversion in vivo (11), it is not taken into account in the calculations of percent P_{FR}. For the purposes of these experiments, it is assumed that the interference filter used in the actinic source causes complete conversion to P_{FR}. However, all values for percent P_{FR} are corrected for the incomplete conversion by the far-red source described above (see 10).

Light Sources. The dim green safe-light consisted of two 15-w green fluorescent tubes (Sylvania F15T8/G) behind 3 mm each of Rohm and Haas Plexiglas, Amber 2451 and Blue 2045. In the manner used, this source caused no detectable conversion of P_R to P_{FR} and had no effect on elongation.

The standard red source consisted of three 15-w red fluorescent tubes (Sylvania F15T8/R) behind a 3-mm thickness of red Plexiglas (Rohm and Haas 2444). Total incident energy at the plant level was about 100 μw/cm². Presumably because of the emission of an appreciable amount of energy at wavelengths above 700 mμ, this source never gave a 100 % conversion of P_R to P_{FR}. Instead, conversion was saturated at about 88 % P_{FR}, irrespective of intensity or duration of illumination (10).

The standard far-red source consisted of five 100-w incandescent bulbs behind 8 to 10 cm of water and a 3-mm thickness of Rohm and Haas V-58015 black Plexiglas. This material has a short wavelength cutoff at 700 mμ, transmitting less than 1 % at or below 705 mμ. Nevertheless, this particular far-red source maintains phytochrome at roughly 5 % P_{FR} (10).

These standard red and far-red sources were thus used to establish the extreme photostationary states of 5 and 88 % P_{FR}. Photostationary states intermediate to these were obtained with the light from incandescent flood lights filtered through 8 to 10 cm of water and 1 thickness of the red Plexiglas described above. In conjunction with this mixed red, far-red source, the segments to be irradiated were placed in small canisters, each equipped with a single Corning colored glass filter or clear Plexiglas. By use of the appropriate filter combinations, photostationary states of 14, 26, 48 and 71 % P_{FR} could be reproducibly established, as tested on *Avena* tissues. These correspond, with no significant differences, to those designated 12, 20, 44 and 64 % on *Pisum* tissue (10).

Results

Initial Photostationary State. The light sources described were used to study the relationship between initial phytochrome steady state and the subsequent elongation of apical coleoptile segments cut from completely dark-grown seedlings. Figure 1 summarizes the results of 5 experiments. All values are normalized to the mean red-irradiated control (88 % P_{FR}) in each experiment. Each point in figure 1 represents the mean elongation of 10 segments. Essentially the same results are obtained with both 5 and 15-minutes irradiation, thereby indicating that the segments are responding to the photostationary state initially established and not simply to the total energy given. This conclusion is identical to that reached with *Pisum* stem segments and may be further confirmed by the same argument that the order of effectiveness of the sources is that of photostationary state and not total energy at any specified wavelength (10, table III). It is evident that all levels of P_{FR} promote elongation, and that this promotion approaches saturation with the conversion of phytochrome to P_{FR} in excess of 48 %.

Effects of Pretreatment of Intact Seedlings. As suggested by the results with *Pisum* stem segments, (10), the fact that elongation approaches saturation with an initial P_{FR} level in excess of 48 % provides

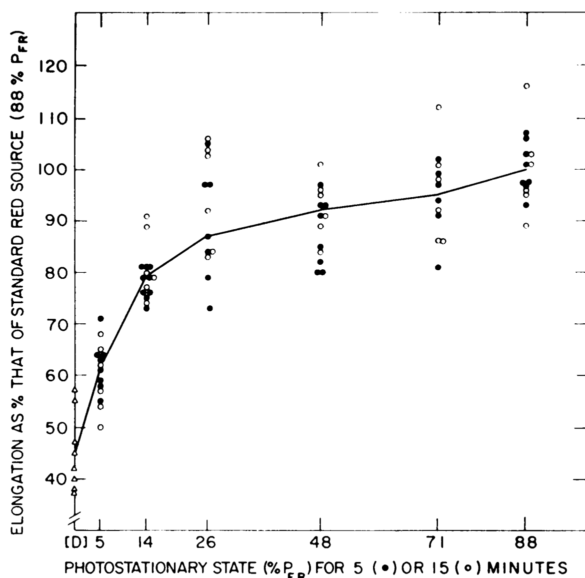

FIGURE 1

FIG. 1. Effects of maintaining the indicated photostationary state (as % P_{FR}) for 5 (●) or 15 (○) minutes on the subsequent elongation of apical coleoptile segments. Each point is the mean of 10 segments, presented as the percentage of the mean red control (88 % P_{FR}) for that experiment. The mean dark elongation for the 5 experiments summarized was 1.07 mm and the mean red elongation 2.29 mm.

no information with respect to the actual amount of phytochrome involved. Experiments were thus begun on material in which the total phytochrome had been reduced by pretreatment of the seedlings with 5 minutes of red light 6 hours before use. Such pretreatment, however, drastically reduced the total elongation and photomorphogenic response of segments cut in the usual fashion. In a typical comparison, segments from totally dark-grown plants elongated about 1.2 mm in darkness and more than 1.9 mm with red light after cutting, while segments from pretreated plants elongated about 0.6 mm in darkness and failed to respond to either red or far-red after cutting.

This loss of growth capacity follows rapidly upon irradiation. For the experiment shown in figure 2, individual flats of seedlings were given 5 minutes red light and returned to darkness for the indicated periods prior to excision of the segments. These were all cut at approximately the same time and incubated in darkness without further treatment. For comparison, control segments were cut from totally dark-grown seedlings and either maintained in darkness or given 5 minutes red light after excision according to the standard procedure. Clearly, the elongation of segments cut as little as 1 to 2 hours after red pretreatment has declined to that of the untreated dark controls. Additional experiments showed that, at any time following the red light treatment, further loss of the growth capacity of segments could be prevented by exposing the seedlings to far-red light. However, while this entire phenomenon is certainly of interest for the control of growth through phytochrome in *Avena*, it appeared to preclude the use of segments from red pretreated plants in the present study.

Photostationary State and Reversibility of the Growth Response. The relationship between phytochrome and segment growth was further investigated by undertaking a comparison of the duration of far-red reversibility with changes in the photometrically measurable phytochrome state, confining all radiation treatments to the already cut segments.

First, excised segments were exposed to 5 minutes red light and then returned to darkness for varying lengths of time before exposure to far-red. All segments were measured 18 to 20 hours after the initial red light treatment. The results of 6 such experiments are summarized in figure 3. The results for each experiment are normalized to the increment of growth promoted by red light in that experiment and each point represents the mean of 30 segments, three 10-segment lots per treatment. These results clearly indicate that reversibility by far-red is completely lost 8 to 9 hours following the initial red exposure. On the basis of this physiological assay, no P_{FR} would be expected to remain in the tissue at this time.

To provide further physiological estimates of

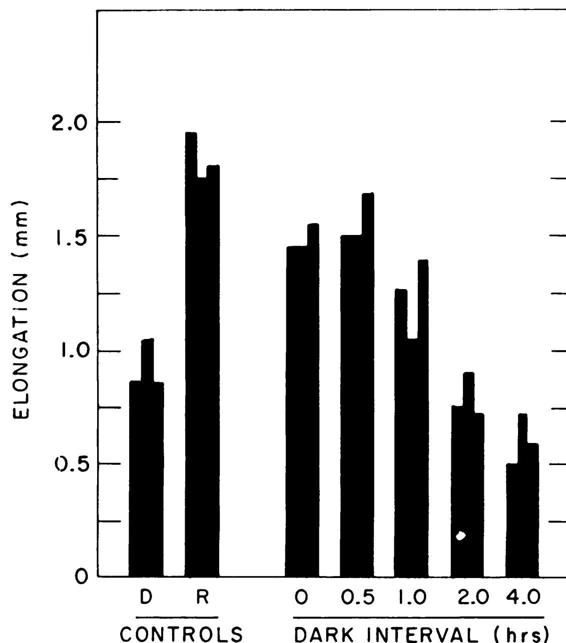

FIGURE 2

FIG. 2. Effect of intervening darkness on the growth response of apical coleoptile segments excised from seedlings pretreated with red light. Controls were cut from completely dark-grown seedlings. See text for details.

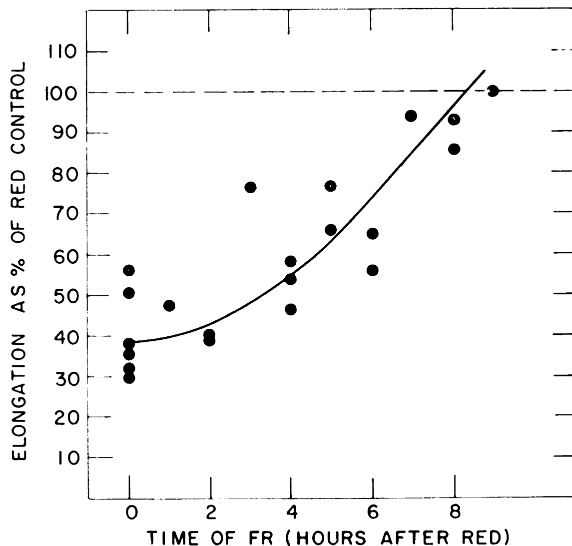

FIGURE 3

FIG. 3. Effect of intervening darkness on the reversibility of the red light response by far-red. Each point represents the mean of 30 segments presented as a percentage of the mean increment promoted by red light in that experiment.

phytochrome state, segments were first exposed to 5 minutes red light and returned to darkness. At 5 and 8 hours following the red treatment, they were exposed to the various photostationary state sources described above. The results of 10 experiments are summarized in figure 4. Controls for each experiment consisted of completely untreated segments and segments which received no treatment other than the initial red light. The results are normalized to the increment of growth promoted by the single, initial red light exposure in each experiment. Each point represents the mean of 30 segments, three 10-segment lots per treatment. The data for 8 hours show no reversibility with either the 26 % P_{FR} filter or the 5 % (standard far-red source), thus confirming the results described above (fig 3). Five hours following the initial red light treatment, only those filters which establish less than about 48 % P_{FR} appear to reverse the red light effect to some extent. On the basis of these data it might be expected that 5 hours following a red light treatment, no more than half the phytochrome is in the far-red form. After 8 hours it would appear to have returned essentially to the dark state, i.e. totally to the P_R form.

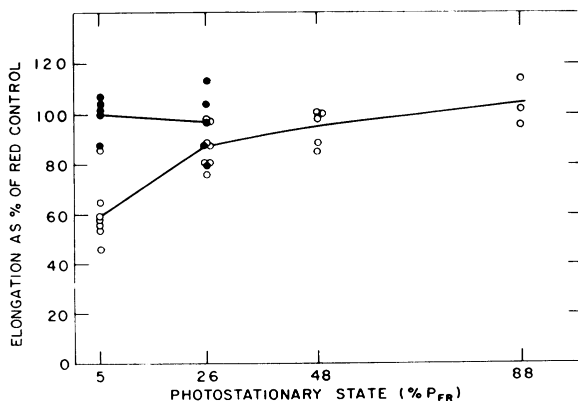

FIG. 4. Reversibility of red sensitive growth by various photostationary states established at 5 (○) and 8 (●) hours. Each point represents the mean of 30 segments, presented as a percentage of the mean increment promoted by red light in that experiment.

For comparison with these predictions, photometric measurements of phytochrome were conducted in segments under conditions similar to those described above. The results with respect to state, percent P_{FR}, are shown in table I. Each value represents the mean of at least 2 determinations, each on separate samples. Five hours following a 5 minute red light treatment, approximately 45 % of the phytochrome is still present as P_{FR}. After 8 hours, a significant proportion of the phytochrome remains as P_{FR}. This latter observation is contradictory to the expectation, on the basis of the above physiological evidence, that phytochrome is totally in the P_R state after 8 hours. However, this was checked directly in 2 of the experiments (B-124, B-125) shown in table I, in which growth tests giving the response shown in figure 4 and the photometric assays were conducted on replicate lots of tissue. The change in total phytochrome in these experiments, though not directly relevant to the question under discussion, may be of interest. Five hours after red light, the total (P_R + P_{FR}) was roughly 20 to 25 % of the initial (hour 0) value, and it declined further to 16 to 20 % at hour 8.

Table I. *Phytochrome State (as percent P_{FR}) in Apical Coleoptile Segments Assayed Photometrically 5 and 8 Hours Following 5 Minutes Red Light*

Expt No.	% P_{FR} 5 hr	8 hr
B-120	50	11
B-122	37	...
B-124	...	22
B-125	48	20
Avg	45	18

Discussion

The elongation of apical coleoptile segments in response to initial conversion of phytochrome is similar to the relationship previously reported for the inhibition of *Pisum* stem segments in that any level of P_{FR} elicits the response and roughly one-half the phytochrome must be converted to give a near maximum effect (10). This suggests a rather straightforward relationship between phytochrome conversion

and physiological response. A similar relationship has been reported for the opening of *Phaseolus* hypocotyl hooks (6). However, results with material pretreated with red light appeared to invalidate any simple interpretation in both the *Pisum* and the *Phaseolus* situations.

Hillman (10) found that segments from red pretreated *Pisum* seedlings, while yielding negative photometric assays for P_{FR}, still responded physiologically as though some 20 % of the pigment were still in the far-red absorbing form. Two possible interpretations were presented, both of which would apply equally well to the results indicated by Edwards and Klein (6) for *Phaseolus* hypocotyl hook opening. The first was that only a small fraction of the total phytochrome present was physiologically active and that the kinetics of dark transformation of the active and inactive fractions differed significantly. The second interpretation was that the apparent decline of P_{FR} in darkness, observed photometrically, represented conversion to a physiologically active, photoresponsive form that could no longer be detected by currently available photometric methods. Neither of these interpretations is required by the results with *Avena,* which seem markedly less contradictory than the others.

Five hours after an initial saturating red light treatment, the physiological and photometric assays are in reasonably close agreement. Both indicate that roughly 50 % of the phytochrome is present as P_{FR}. This observation, considered along with the close correlation between initial state and elongation, suggests that in the coleoptile response the photometrically assayable phytochrome maintains a close relationship to the physiological system for up to 5 hours. However, at 8 hours, the agreement is no longer so good. At that time, further elongation of the segments can no longer be prevented by far-red, although a significant proportion of the photometrically detectable phytochrome assays as P_{FR}.

Explanation of the 8-hour disagreement on the basis that elongation has proceeded to its maximum by then is easily invalidated: the growth curve for such segments (12) shows that 50 % or less of the elongation measured after 20 hours has been accomplished after 8. The most probable explanation is simply that by 8 hours the nonphotochemical reaction sequence initiated by P_{FR} has progressed far enough so that P_{FR} is no longer required for its maintenance. The assumption of such an escape from photochemical control is basic in the photoperiodic literature (1). What is most important is to note that the situation here is the precise opposite of that previously observed in *Pisum* (10) and *Phaseolus* (6) since, in these, physiological responses presumed to be dependent upon P_{FR} were obtained in its apparent photometric absence. Hence the relationship between phytochrome state and physiological response in *Avena* is the most rational, or at least the most explicable, yet observed. More detailed investigations of this system may of course destroy this conclusion; for example, experiments starting with lower initial degrees of conversion might provide a more severe test of the apparent agreement between photometric and physiological assays. But it is at least clear that experiments of the kind that uncovered a contradictory situation in *Pisum* have failed to do so in *Avena.*

While direct comparison of the *Avena* and *Pisum* results might appear unwise, since in the latter the light treatments were not confined to the excised segments, the *Phaseolus* system, handled in this regard as is the *Avena* (6, 14), nevertheless gives results similar to *Pisum* and unlike *Avena.* This suggests strongly that the operative difference, in fact, is one between coleoptile and dicotyledonous stem tissue. That there are differences between these 2 types of tissue in the kinetics of phytochrome transformations has been reported previously (11). These differences may thus be reflected in the relationship between photometric and physiological estimates of changes in phytochrome state, though by what underlying mechanism it seems futile to speculate here. It is particularly surprising that *Avena,* in which no true reversion of P_{FR} to P_R is detectable, should provide an apparently more rational relationship than the legumes, in which reversion may well occur (11), in view of the general assumption (e.g. 1) that reversion is the physiologically significant process. The determination of such relationships in other tissues, exhibiting various combinations of reversion and decay, now seems desirable.

Finally, the effect of light pretreatment of the *Avena* seedling on the subsequent growth of coleoptile segments merits further investigation. The phenomenon is certainly phytochrome-mediated, since at any time following the red light treatment, further loss of the growth capacity may be prevented by exposing the still intact seedling briefly to far-red light. Whatever process is initiated in the seedling by red light apparently remains under continuous control of the photoreceptor until completion, but may be interrupted by either excision or far-red light. Briggs (2) has found that red light induces a rapid decrease in the amount of diffusible auxin in the apices of both *Zea* and *Avena* coleoptiles. Excision of the apex following red light prevents the further loss of auxin, and the kinetics for both phenomena are very similar. The suggestion may be made that the loss of growth capacity in intact *Avena* coleoptiles is related in some way to the observed decrease in diffusible auxin. There may also be a close relationship between these phenomena and the effect of red light pretreatment on *Avena* geotropism reported by Wilkins (13).

Acknowledgment

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