Persistent Photoreversibility of Leaf Development
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Abstract. Far red light reversal of red light induced leaf expansion and enzyme changes were investigated in seedlings of Phaseolus vulgaris var. Black Valentine. In etiolated plants growth, anthocyanin accumulation and increases in glyceraldehyde-3-phosphate dehydrogenase and glycolic acid oxidase activities induced by a 10 min red irradiation were stopped by a 7 min far red irradiation given 17, 24, or 48 hr after activation. Etiolated seedlings illuminated for 24 hr with white light and seedlings grown in continuous light remained sensitive to far red reversal. This suggests that the far red sensitive receptor does not decay with time but remains associated with the site of its regulatory functions.

In considering models for the physiological action of phytochrome the stability of the "active" form of the pigment plays a crucial role. Designated as P730 this form has been considered alternately as rapidly reverting to P660 (1), decaying (9) or both (2). On the other hand Downs reported in 1955 that far-red reversibility of red light activated leaf elongation was lost very slowly. In 8 hr only about 20% of the reversibility was lost when adjustments were made for the intervening growth of the seedling (3). This points to an extremely stable "active" photoreceptor.

Recently we presented evidence (7) that light-activated increases in total protein and ribulose diphosphate carboxylase activity of etiolated bean leaves remain partially reversible for over 24 hr. The remarkably long-lived susceptibility of developmental changes to reversal has interesting implications for models of growth regulation by phytochrome, particularly when contrasted with the lability of spectrophotometrically detectable P730 (4, 12). We, therefore, undertook an exploration of the limits within which red light activated developmental responses of bean leaves remain far red reversible.

Materials and Methods

Growth and Illumination of Seedlings. Seeds of Phaseolus vulgaris var. Black Valentine (the generous gift of Charter Seed Company, Twin Falls, Idaho) were germinated in vermiculite in a dark growth room at 22°. Ten min of promoting irradiation was provided by 3 General Electric red fluorescent tubes through a one-eighth inch thick Rohm and Haas Red 2444 Plexiglas filter. Continuous irradiation was in a 26° growth chamber fitted with warm white fluorescent tubes supplemented by incandescent bulbs. Irradiation with far red light was by four 150 W Sylvania flood lights above a 6 cm water heat filter and a one-eighth inch thick Rohm and Haas V58015 Plexiglas filter. Exposure time for far red irradiation was 7 min. Distance of the lights from the plants was 25 cm for the red source and 40 cm for the far red source.

Sampling. For all measurements a sample of 40 or 60 primary leaves taken from half as many plants were used. Repeat experiments showed variations in absolute values but not in patterns of response. Values presented here are representative of 3 or more separate experiments.

Enzyme Assays. Preparation of homogenates and details of the assays were as previously published (7).

NADPH-linked glyceraldehyde-3-P dehydrogenase was determined by a modification of the method of Wu and Racker (19). Glycolic acid oxidase was determined according to the methods of Tolbert and Burris (18) and Zelitch and Ochoa (20).

Protein. Trichloroacetic acid (5 %, v/v) precipitable protein was determined by the methods of Lowry et al., (14).

Anthocyanin Content. Determination of relative anthocyanin content of hypocotyl epidermis was carried out on ethanolic HCl (1 % v/v) extracts. Ten cm segments of hypocotyls taken 4 cm below the hypocotyl hook were extracted 3 times. The optical density of the extract at 540 nm was determined. From this data relative anthocyanin content was calculated and expressed as OD ml/cm². This represents the optical density of an extract containing the anthocyanins of 1 cm² of epidermal tissue in 1 ml.

Results

A measure of leaf growth is provided by changes in the fresh weight and protein content of the organ.
Determinations of changes in enzymatic activities permit an even more sensitive monitoring of development, particularly when we are seeking to measure alterations in the rate of growth. The patterns of fresh weight, protein, and several enzyme activity changes in the leaves of 7-day-old etiolated bean seedlings were previously described (7). Several investigators reported that a brief illumination is sufficient to trigger an increase in size and NADPH-linked glyceraldehyde-3-P dehydrogenase (GPD) activity of the leaves (7, 15, 16). In our present study the most rapid increase in GPD activity took place between 17 and 24 hr following illumination. Treatment with far red light 17 hr after an initial promoting illumination prevented this increase (Fig. 1). There was an immediate and drastic drop in the increase of enzyme activity produced by the far red treatment even though it was given at the onset of the rapid changes in enzyme activity.

We noted previously that after red light treatment the activity of another enzyme, glycolic acid oxidase (GAO), increased for a longer period than that of GPD. Yet at a time when one-third or even three-quarters of the potential response had already occurred far red light could prevent further substantial increases in GAO activity (Fig. 2).

Such persistent sensitivity to reversal made us wonder whether the 10 min of initial red illumination while adequate as a trigger might be insufficient to stabilize or fix the developmental program at its new, more active level. If so then a more prolonged initial illumination might provide greater stabilization and abolish susceptibility to far red reversal. To test this a 24 hr light treatment was selected, not only because of its total length but also because it spans the period during which rapid changes begin. Results presented in Fig. 3 demonstrate that 24 hr of continuous illumination did not abolish far red depression of subsequent activity increases in the dark. Thirty hr after far red irradiation GPD activity was almost half of the unreversed control. The transient dip in activity which follows return to darkness in both reversed and unreversed plants remains an unexplained but reproducible phenomenon.

Because the preceding experiments were performed on etiolated seedlings there remained the possibility that their prolonged growth in darkness contributed to the lability of light stimulated developmental change. We therefore germinated seeds in continuous light and grew the seedlings under these conditions for 8 days. They were then divided into 3 groups. One group was left in constant light, another was transferred to the dark and the third was briefly irradiated with far red light and then transferred to the dark. The growth and GPD activity changes of the 3 groups are presented in Fig. 4. The continued effectiveness of far red light

![Fig. 1. Reversibility of increase in NADPH-linked glyceraldehyde-3-P dehydrogenase activity induced by brief red irradiation in leaves of 7 day old etiolated seedlings. All plants kept in the dark except during illumination as follows: 10 min red light at 0 hr (L); 10 min red light at 0 hr, 7 min far-red light at 17 min (FR); no illumination (D). Reaction mixture contained 0.1 ml 0.4 M tris buffer pH 8.5, 0.1 ml 0.1 M MgCl2, 0.1 ml 0.04 M cysteine, 0.02 ml phosphoglyceric acid phosphokinase, 0.02 ml 0.01 M NADPH, 0.48 ml water, 0.1 ml extract. Following a 4 min preincubation the reaction was started by the addition of 0.04 ml 0.1 M ATP and 0.04 ml 0.01 M 3-PGA. Optical density changes at 340 nm were recorded for 5 min.](image1)

![Fig. 2. Reversibility of increase in glycolic acid oxidase activity induced by brief red irradiation in leaves of 7 day old etiolated seedlings. All plants kept in the dark except during illuminations as follows: 10 min red light at 0 hr (L + D); 10 min red light at 0 hr, 7 min far-red light at either 24 hr (L + D24 + FR) or 48 hr (L + D48 + FR); no illumination (D). Oxygen uptake was determined manometrically. Reaction mixture contained 1.5 ml 0.1 M phosphate buffer pH 8.0, 0.3 ml water, 0.1 ml 0.003 M FMN, 0.1 ml 0.3 M KCN, 0.5 ml 0.04 M potassium glycolate, 0.5 ml extract. Volume changes were recorded at 25° for 30 min.](image2)
in halting further growth and enzyme accumulation even after prolonged continuous illumination is evident.

Far red inhibition of other morphogenic changes is equally striking. The growth of the petiole and unfolding of leaves can be inhibited even after growth in continuous light. These events are more difficult to describe in a quantitative way but the extent of the inhibition may be seen in the silhouettes repro-

Table I. Far-red Inhibition of Anthocyanin Accumulation by Hypocotyl Epidermis in the Dark

<table>
<thead>
<tr>
<th></th>
<th>24 hr light, 48 hr dark</th>
<th>24 hr light, 7 min far-red, 48 hr dark</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface area of hypocotyls, mm²</td>
<td>1187</td>
<td>1149</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1231</td>
<td>1212</td>
<td></td>
</tr>
<tr>
<td>Anthocyanin content, OD</td>
<td>2.80</td>
<td>1.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.04</td>
<td>1.89</td>
<td></td>
</tr>
<tr>
<td>Anthocyanin per unit surface area</td>
<td>0.0236</td>
<td>0.0156</td>
<td>33.9</td>
</tr>
<tr>
<td>OD ml/cm²</td>
<td>0.0247</td>
<td>0.0164</td>
<td>33.6</td>
</tr>
</tbody>
</table>

1 Growth conditions and light treatment as in Fig. 5.
2 Surface area of ten 10 cm long hypocotyl segments taken starting 4 cm below hypocotyl hook.
3 OD at 540 nm of HCl:ethanol (1% v/v) extracts containing anthocyanins of 10 segments in 10 ml.
4 OD of an extract calculated to contain the anthocyanins of 1 cm² per ml.
duced in Fig. 5. These plants received treatment identical to those in Fig. 3, that is, they were exposed to white light for 24 hr and were then either placed directly into the dark or were given a short far red irradiation and then placed in the dark. Two days later the leaves were detached, arranged on a sheet of paper, covered by a glass plate and their outlines reproduced on an office photocopying machine. The photocopy was then photographically intensified. The inhibition of expansion, petiole elongation and the change in the angle between the blade and the petiole are evident. Equally marked differences in the unfolding of the blade along the midrib cannot be seen because of the necessity to compress the leaves under the glass plate.

Continued anthocyanin accumulation by the epidermal layer of hypocotyls also remains subject to far red control. Table 1 presents data indicating that 48 hr after light treatment far red reversed epidermis accumulates one-third less anthocyanin than the unreversed control.

Finally we had to assure ourselves that the uniformly negative effects of far red irradiation were not an irreversible debilitation of the seedlings by a non-specific inhibitory treatment. To show that far red treated seedlings are still capable of normal growth responses a second promoting red irradiation was given a group of seedlings which had begun to show the effects of far red reversal. Fig. 6 shows that red light re-starts the growth of leaves indicating their continued responsiveness to growth promoting illumination.

Discussion

Phytochrome mediated developmental responses appear to be more sensitive to photoreversal than the spectrophotometrically detectable pigment (27,30) levels would indicate (9). There is, nevertheless, insufficient information available on the degree of stability of the physiologically active state and, by implication, of 17,30. In our previous communication (7) we reported developmental changes which remained partially reversible for at least 48 hr. This was in contrast to the limits of reversibility reported for several other systems: 30 to 60 min for flower induction in Xanthium (6), 8 hr for pea stem elongation (11), and 20 hr for lettuce seed germination (3). There were earlier indications, however, that escape from reversibility may be slower in leaf expansion (5) and hypocotyl hook-opening in bean (12).

Several possible mechanisms may operate in determining the length of the period between initial stimulus and escape from reversibility. One is the disappearance of the far-red absorbing pigment by whatever route. Because at present we cannot measure levels of physiologically active 17,30 we can only state that in bean leaves complete disappearance

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24 HRS LIGHT 24 HRS DARK

Fig. 5. Inhibition of morphological changes induced by a 24 hr illumination. Seven day old etiolated seedlings were illuminated for 24 hr with white light. One group was returned directly to the dark (left), a second group was irradiated with far-red light for 7 min and then returned to the dark (right). Forty-eight hr later the outline of 20 leaf pairs was recorded with a photocopying machine.

24 HRS. LIGHT-PR. 48 HRS. DARK

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GPD increases are abolished almost immediately if the reversal is effected at the onset of the response. In all experiments in which GPD was measured changes in the slope of increase were noticeable within 6 hr. Decrease in glycolic acid oxidase activity following inhibition may be accounted for in 2 ways: either the rate of enzyme synthesis remains unchanged for about 12 hr and then levels off, or, as we represented it in Fig. 2, there is an immediate reduction in the rate of increase resulting in slower accumulation which in 24 hr is equivalent to 12 hr of unimpaired production.

By increasing the period of promoting irradiation from 10 min to 24 hr or continuous light we have demonstrated that the far red sensitivity of subsequent growth in the dark cannot be abolished. There is no locking of the response into a "light pattern". Perhaps the most dramatic illustration of the continuous regulation of growth by phytochrome may be seen in Fig. 4. After a prolonged period of normal growth and enzyme production, a brief irradiation with far-red light drastically reduced further growth and completely prevented the doubling of GPD activity in the dark.

Acknowledgments

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Literature Cited


