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Evidence for a Phytochrome-Mediated Phototropism in Etiolated Pea Seedlings[1]

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ABSTRACT

Entirely etiolated pea seedlings (*Pisum sativum*, L. cv Alaska) were tested for a phototropic response to short pulses of unilateral blue light. They responded with small curvatures resembling in fluence-dependence and kinetics of development a phytochrome-mediated phototropic response previously described in maize mesocotyls. Irradiations from above with saturating red or far-red light, either immediately before or after the unilateral phototropic stimulus, strongly reduced or eliminated subsequent positive phototropic curvature. Only blue light from above, however, entirely eliminated curvature at all fluences of stimulus. It is concluded that the phototropism is primarily a result of phytochrome action.

Phototropism in both monocots and dicots has classically been considered to result from differential growth in response to blue or ultraviolet radiation (4, 7). The blue and UV-A light photoreceptor, sometimes called cryptochrome, responsible for phototropism in a wide variety of plants and fungi, has a characteristic action spectrum that declines essentially to zero for wavelengths longer than about 500 nm (2). The long wavelength regions of the spectrum are generally found to be phototropically inactive, although they are well known to have a multitude of growth and developmental effects in all classes of plants through their photoconversion of the pigment phytochrome (15).

The chromophore of phytochrome absorbs primarily in the red and far red regions of the spectrum, for the P_r[3] and P_{fr} forms, respectively, but it also has significant absorption in the blue and UV-A (5). Both the P_r and P_{fr} forms of phytochrome absorb light in the blue part of the spectrum, but P_{fr} absorbs somewhat more strongly than P_r, so that only a small amount of P_{fr} is maintained in photostationary equilibrium under blue light (17). This amount is enough to have several effects on the metabolism and development of a plant (5, 14), however. So although red light does not cause phototropism directly through the excitation of cryptochrome, blue light may elicit phytochrome-mediated responses.

Phototropism results when the growth rates of the shaded and irradiated sides of an unilaterally irradiated plant differ. It is well known that phytochrome can act to inhibit the straight growth of etiolated seedlings of both monocots and dicots (wheat, 3; corn, 19; pea, 16). If the optical properties of a unilaterally irradiated seedling are such that a gradient of light is produced across its stem within a range of fluences where the phytochrome-mediated inhibition of growth is not yet saturated, a difference in inhibition of growth across the stem should result, leading to a phytochrome-mediated phototropism.

Iino, *et al.* (9) recently reported what was clearly a phytochrome-mediated phototropism in corn mesocotyls elicited either by blue light or red light. The fluence-response curves they presented show two maxima of curvature, corresponding to the two phases of the fluence-response curve for inhibition of mesocotyl straight growth by phytochrome (12). In this paper we describe a similar phytochrome-mediated phototropism elicited by blue light in the epicotyls of totally etiolated peas.

MATERIAL AND METHODS

Plant Material

Pea seeds (*Pisum sativum* L. cv Alaska) were surface sterilized for 15 min in a 20% solution of commercial bleach (All Pure Chemical Company, Tracy, CA). They were rinsed well in deionized tap water and sown in white light onto narrow trays (22.5 × 7.5 × 5.5 cm) containing wet vermiculite, 30 to 40 seeds per tray, and covered with about 1 cm of wet vermiculite. The trays were placed into opaque grey plastic boxes (48 × 60 × 25 cm) with opaque tops. The boxes were wrapped in two layers of thick black cotton cloth and kept in a light-tight dark room at 24°C. The seedlings received no light of any kind after planting until their experimental treatments on the fifth day after planting. At this time their epicotyls ranged in length from 4 to 9 cm and had hooks at various angles, with yellow buds 4 or 5 mm long.

Light Sources and Irradiation Procedures

For irradiations with unilateral blue light, the light source used was a theatrical lamp, with a white GE FEL 1000 W quartz iodide bulb, hung vertically over a table. The beam was filtered through about 5 cm of water, a blue glass filter (Corning B5-60, 5 mm thick), and 1 cm of a 10% $CuSO_4$ solution held in a clear acrylic plastic box. Neutral density filters were constructed of white paper or white cheesecloth and calibrated on the light source. Light passing through the filters was diverted with a mirror through a black cardboard tunnel to strike the plants along their whole length from the side. The resulting beam was a rectangle with a width of 20

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[3] Abbreviations: P_r, the inactive form of phytochrome; P_{fr}, the active form of phytochrome; P_{tot}, total phytochrome.

cm and a height of about 10 cm. The beam's intensity varied by approximately 5% across its width and height. Photon fluence rates were measured directly using a portable quantum spectrophotometer (Li-1800, LiCor Corp.). Fluence rates under 0.2 μmol m^{-2} s^{-1} were calculated from photovoltmeter readings (model 520 M, Photovolt Corp.), assuming an average wavelength of 450 nm for the beam. An Eppley 8-junction bismuth-silver thermopile in conjunction with a 150B μV ammeter (Keithley Instruments, Inc.) was used to calibrate the photovoltmeter. Similar calculations at higher fluence rates agreed well with direct photon fluence measurements.

In several experiments, 5 min of red, blue, or far-red light was given to the plants from the top either immediately before or immediately after the blue phototropic stimulus. The transition time in the dark between irradiations was 5 s or less. For these experiments, the red light source was a red fluorescent bulb (Sylvania Red F20T12/R, 20W). The light was filtered through one layer 3 mm thick of acrylic plastic (Shinkolite A102, Argo Plastics). This arrangement gave a fluence rate of 8.0 μmol m^{-2} s^{-1} at the height of the plant tips. Consequently, the plants received a total fluence of 2.4×10^3 μmol m^{-2}. The far-red light source for these experiments was the incandescent lamp previously described, with the tunnel and mirror arrangement removed. The light was filtered through a plastic filter (Rohm and Haas FRF 700) to give a far-red beam with a fluence rate of 2.6×10^3 μmol m^{-2} s^{-1}, so the plants received a total of 7.8×10^5 μmol m^{-2}. The blue light source for pre- or poststimulus treatments was a blue fluorescent bulb (GE 20 W, F20T12/B). The beam was filtered through one layer of Cinemoid plastic (No. 857). The fluence rate of this source was 11.8 μmol m^{-2} s^{-1}, for a total fluence over 5 min of 3.54×10^2 μmol m^{-2}.

In position transducer experiments the straight growth of epicotyls was measured before, during, and after irradiation with 5 min of red or far-red light. The position transducer apparatus (described below) was set just inside the irradiation beam of the incandescent lamp. The beam was filtered through the far-red or red acrylic filters just described. The red filter on this lamp produced a beam with a fluence rate of 4.0×10^3 μmol m^{-2} s^{-1}. The plant was not moved during the course of the experiment. During a 5 min irradiation with red light, a plant received a total fluence of 1.2×10^6 μmol m^{-2}.

Determining the Phototropic Response

To induce phototropic curvature, a 30 s pulse of blue light of the appropriate fluence rate was given to the plants unilaterally. The maximum fluence used, 5.6×10^2 μmol m^{-2} s^{-1}, was determined by the fluence the lamp delivered in 30 s without neutral density filters. The angle between the plane of the hook and the plane of irradiation was not controlled. After being irradiated, plants were held in light-tight boxes until harvest. Each tray was harvested in white light in less than 5 min. At the harvest, all plants showing more than approximately 20° curvature localized to within a 1 cm section of the epicotyl outside the growing zone were discarded. This was considered a valid selection because it was found to reduce the variance around the means of curvature in different treatments, but not to change the means themselves. All plants that were not vertical at the base were also discarded. Of the remaining 10 to 15 plants, the tallest 8 were chosen. The chosen plants were cut off at the level of the vermiculite and taped to a piece of Plexiglas with the plane of irradiation parallel to that of the plastic. Their hooks were removed and they were photocopied.

Curvature was defined as the angle on the photocopied image between lines drawn parallel to the long axis of the epicotyl at the base and at the tip. Angles were measured on a digitizer (HP 9111A Graphics Tablet, Hewlett-Packard, Sunnyvale, CA). Totals, averages, standard errors of means and variances per tray were given by the digitizer program. Another person was occasionally asked to select plants or draw and digitize angles from photocopied images without knowing what treatment the plants had been given. These tests revealed no particular direction or magnitude of experimenter bias. In an F_{max} test for homogeneity of variance, no significant differences were found for the same treatment either within or between days, so data from experiments on different days were pooled.

Measurements of Straight Growth

To measure the straight growth rates of etiolated peas, and their responses to red and far-red light pulses, pea seeds were grown as before, but were sown individually into 30 mL glass beakers filled with damp vermiculite. After 5 d of growth plants were chosen for experiments using an infrared light source and an infrared sensing scope described previously (10). The infrared irradiation was always less than 15 s in duration. Only straight plants with hooks sufficiently bent to retain a string were used.

Plants were attached in the total darkness to a linear position transducer core (Schaevitz, Pennsauken, NJ) by a string, with a slip-knot tied gently around the neck of the hook. The string was held taut with a counterbalancing weight of 3 g over a knife-edge fulcrum. Motion of the core of the position transducer was registered as a change in voltage output and was recorded on a chart recorder. The growth rate of the pea being observed was calculated over 5 min intervals from the slope of the chart recorder output. The plants often failed to grow for up to 10 min after being handled, so approximately the first 15 min of each growth tracing was discarded. Light treatments were given 120 to 140 min after attachment.

Measurements of Photostationary Levels of Phytochrome

For each of the light sources used, the photostationary level of etiolated pea phytochrome was measured. Peas were grown as for a phototropic experiment and were exposed from above to the light source to be tested. Neutral density filters were not used. Buds, along with the connecting top 0.5 cm of hypocotyl, were harvested under the same light, chopped on ice, and transferred on ice under black cloth to a Ratiospect (Agricultural Specialties, Inc.). This instrument measured the difference of the absorbances at 730 and 800 nm. Each sample, still on ice, was then driven to photostationary equilibrium with a red light source, measured, then driven to photostationary equilibrium with a far-red light source and measured again. This procedure provided an internal calibration for each sample. On the basis of its transmission spectrum, the fluorescent red light source with red filter described

previously was considered to yield, at photostationary equilibrium, a P_{fr}/P_{tot} ratio of 0.80 (13). Similarly, the far-red source we used to establish endpoints in these measurements—a slide projector with a GE projection lamp bulb (500 W, 120 V), filtered through a Corning 7-69 glass filter—was assumed to yield at photostationary equilibrium 0.03 P_{fr}/P_{tot}.

RESULTS

The phototropic fluence-response curve in Figure 1 was obtained with blue light. It shows two maxima, one at approximately 1.6×10^{-2} μmol m^{-2}, and the other at approximately 10 μmol m^{-2}. The response in the range between the two maxima does not quite return to zero. Curvature was not found in the fluence range between 10^{-3} and 6.5×10^{-5} μmol m^{-2}. The fluence range above approximately 7.9×10^{2} μmol m^{-2} was not investigated. Following Iino *et al.* (9), we have called the more sensitive peak of curvature 'first positive' and the less sensitive peak 'second positive.'

The two maxima develop over different time courses (Fig. 2). The first positive curvature is detectable within 30 min after the stimulus, but there is a period of about an hour after the stimulus before second positive curvature begins to be detectable. This is exactly analagous to the time courses of development of curvature found by Iino *et al.* (9) in the phytochrome-mediated phototropism in maize mesocotyl. Both first and second positive curvature reached a maximum at about 90 min.

Figure 3 gives the results of experiments testing the effects of red, far-red, and blue light pre- and poststimulus irradiations on plants given a phototropic stimulus at either the first or second positive peak fluence. For these experiments, as well as for the linear position transducer experiments, the fluence rates of the different light sources were considered to be more or less unimportant, as long as they were large. We only hoped, in a short irradiation period, to saturate as quickly as possible the phytochrome conversion obtainable with that particular light source. Red light pre- or postirradiations alike reduced or eliminated curvature in response to first or second positive range stimuli. Far-red light pre- or postirradiations reduced subsequent curvature about 50% for plants given a stimulus in the first-positive range, but entirely eliminated curvature in plants given a stimulus at the second positive peak fluence. Blue light given before or after the stimulus eliminated both first and second positive curvature, and may even have induced a small negative curvature.

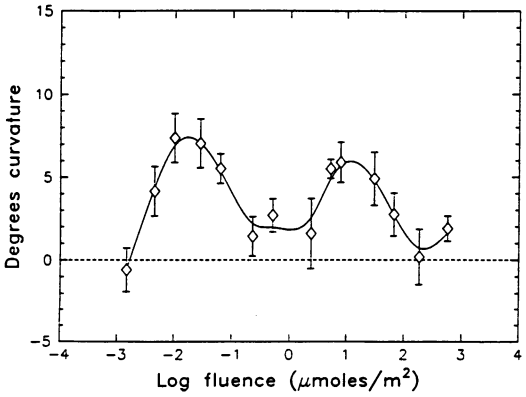

Figure 1. Fluence response to unilateral blue light. Error bars represent ± SE, and the number of plants measured for each point ranged from 16 to 95, with an average of 37. The time between irradiation and harvest was between 2.5 and 4 h, a period during which both first and second positive curvatures were stable (see Fig. 2). The line shown was fitted by eye to fall within the standard errors for each point. The data for this graph were gathered over a 6 month period.

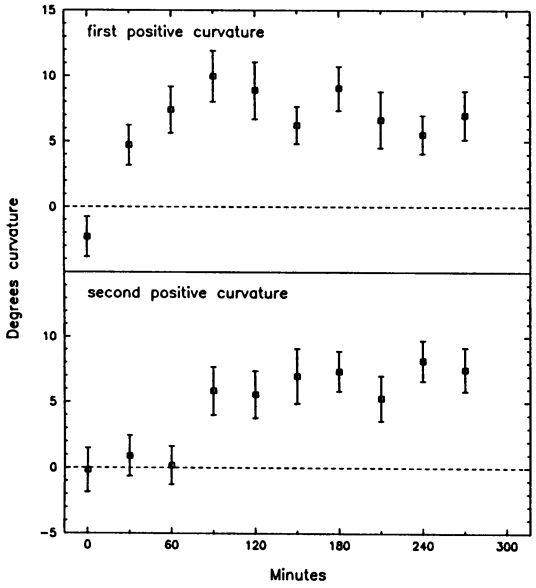

Figure 2. Development of first and second positive curvatures. For the time course of first positive curvature, a fluence of 6.2×10^{-2} μmol m^{-2} was given to the plants in a 30 s pulse. For second positive curvature, a fluence of 7.8 μmol m^{-2} was given in a 30 s pulse. Error bars represent ± SE, and 24 plants were measured for each point.

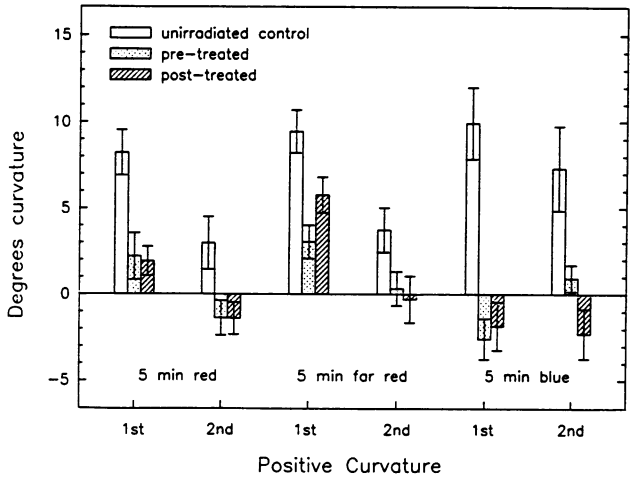

Figure 3. Effects of various light treatments on first and second positive curvature. Five min of red, far-red, or blue light were given as a pre- or posttreatment to plants receiving a phototropic stimulus in either the first positive (1st) or second positive (2nd) range of the fluence-response curve. The same fluences of light used to elicit first and second positive curvatures in the experiments reported in Figure 2 were used in these experiments. Error bars show ± SE. Sixty-three or 64 plants per point were measured for each red light experiment and the controls pertaining to it; 24 plants were measured per point in experiments where a far-red treatment was given; 32 plants per point were measured in the blue light pre- or posttreatment experiments.

It seemed possible that this elimination of curvature in response to red or far-red preirradiation resulted from a dramatic decrease in growth rate, so we tested the effects of red and far-red light irradiation on the peas' straight growth using a position transducer. The growth rate traces for individual control plants always showed oscillations of about 10% in either direction around a slowly decreasing growth rate. These oscillations had a period of about 1 h, and may have reflected nutation of the pea superimposed on a slowly decreasing straight growth rate (11). The control curve in Figure 4 shows the average of five unsynchronized plants, and so does not show an easily discernible oscillation. Plants receiving a 5 min irradiation with red light showed a significant drop in growth rate within 15 min of the start of the treatment. Plants receiving 5 min of far-red light reacted nearly identically. Both traces show a small shoulder at about 60 min after treatment, and both have a small but significant recovery in growth rate (10–20%) 220 to 230 min after the start of irradiation. Preliminary experiments indicate that these initial events represent a nutational response to red or far-red light, superimposed on a true growth decrease (data not shown). The two traces only diverge from each other at about 90 min after the beginning of irradiation. Plants treated with far-red light were, over a 5 h period after treatment, reduced in rate to about 50% of the preirradiation average, whereas over the same time period the red-treated plants slowed to about 20% of the pretreatment average.

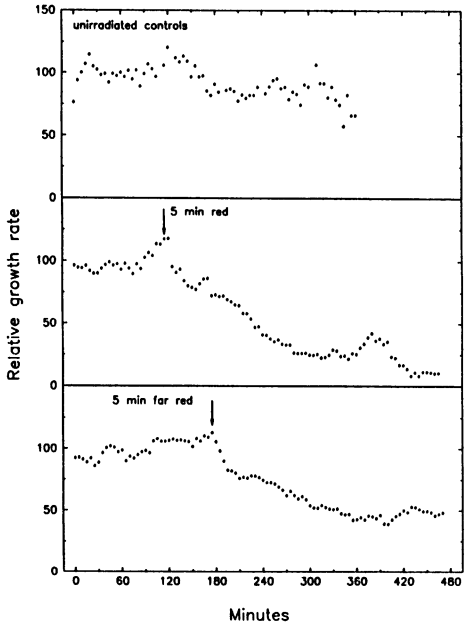

Figure 4. Effects of red or far-red irradiation on growth rate. Each curve represents the average of 5 plants. The unirradiated controls were normalized to the average growth rate over the first 120 min. The irradiated plants' traces were normalized to the average preirradiation growth rate. Each point represents the average rate of growth over a 5 min interval. The arrows show the 5 min intervals in which irradiations began. SEs were not calculated because the sample size was small, but the range of normalized values for each point in irradiated plants rarely exceeded 20% of the preirradiation value. The range of values for the unirradiated controls varied somewhat more than that, up to about 50% of the average growth rate over the first 120 min. Generally the ranges of values increased over the time the plants were attached to the position transducer.

Measurements of the photostationary ratios of P_{fr}/P_{tot} were performed to determine the relative degree to which each light source converted phytochrome. The results are shown in Table I. As expected, the fluorescent light source with red filter was the most effective of the sources at converting phytochrome to P_{fr}. The incandescent blue light used to deliver the phototropic stimuli yielded a ratio of 0.34 after 30 min when unattenuated, indicating that the amount of phytochrome conversion obtained in 30 s of unattenuated light was very small.

DISCUSSION

We suggest that the phototropism described in these experiments was primarily phytochrome-mediated, because the fluence-response curve is similar in shape, in fluence threshold, and in timing of development to that reported by Iino *et al.* (9); also because irradiations by red and far-red light either immediately before or immediately after the blue light stimulus suppressed or eliminated the phototropic response. The phytochrome-mediated phototropism in maize mesocotyls probably results from a gradient of phytochrome-mediated inhibition of growth across the mesocotyl. Inhibition of pea (16) and cucumber (8) seedling elongation by red light has been reported, and is considered to be phytochrome-mediated by virtue of its action spectrum and photoreversibility, but it has not been characterized in either plant under the conditions of low phytochrome photoconversion we investigated.

A phytochrome-mediated phototropism has not been described in peas previously. There are at least three reasons why. First, fluences of red light used in such experiments may have been of such a magnitude that the red light distributed across the stem of the seedling inhibited growth equally on both sides. Second, red light may not be able to create a steep enough gradient of P_{fr} across the hypocotyl of a pea to give detectable bending, even within an appropriate fluence range, since it is not as highly scattered and absorbed by plant tissues as is blue light. Third, peas which have been exposed to light

Table I. *Determinations of the P_{fr}/P_{tot} Ratios Produced by the Different Light Sources Used*

The ratios reported here were calculated by linear extrapolation between two reference values. The fluorescent red source was assumed, on the basis of its transmission spectrum, to yield the maximum obtainable ratio P_{fr}/P_{tot} of 0.80 and served as the upper reference value. An incandescent far-red light source, described in "Material and Methods," was assumed to yield a ratio of 0.03. The error in these measurements, determined by measuring the apparent ratio P_{fr}/P_{tot} in unirradiated controls, was ±0.06.

Light Source	Duration of Irradiation	P_{fr}/P_{tot}
	min	
Incandescent red (4.0×10^3 μmol m^{-2} s^{-1})	5	0.54
Incandescent far red (2.6×10^3 μmol m^{-2} s^{-1})	5	0.04
	30	0.04
Incandescent blue (18.7 μmol m^{-2} s^{-1})	5	0.28
	30	0.34
Fluorescent blue (11.8 μmol m^{-2} s^{-1})	5	0.19
	30	0.29

during germination and early growth develop a cryptochrome-mediated phototropism of up to 25 ° of curvature (1), which could easily mask the small phytochrome-mediated phototropism if the peas used were not completely etiolated.

Iino *et al.* (9) found that blue light gave approximately twice the curvature in maize mesocotyls that red light gave. This is because the fluence-rate gradient of blue light through the mesocotyl is about twice as steep as that for red light (12). Seyfried and Schäfer (18) found that the local fluence rate of blue light through increasing thicknesses of etiolated cucumber cotyledon also declines more steeply than the local fluence rate of red light, and consequently creates a steeper gradient of P_{fr} within the cotyledon. If the wavelength dependence of internal light gradients is similar for pea epicotyls, cucumber cotyledons and maize mesocotyls, one might expect to see only about 3° of curvature in response to red light. Detectable curvature was not inducible in this system with unilateral red light in the fluence range 0.31 to 1.2×10^5 μmol m^{-2}. This range, accounting for the approximately 100-fold greater phytochrome conversion effectiveness of red light over blue light (17), should have spanned at least the second positive peak of curvature. Attempts to increase the response magnitude by rotating plants on a clinostat following unilateral red-light irradiation have proved inconclusive. Untreated peas respond to as little as 4 h on the clinostat by greatly increasing their variability of curvature around vertical.

A phototropic stimulus in the first positive range preceded or followed by 5 min of red or far-red light always left a residual curvature. This residual response is difficult to explain, and may represent a small cryptochrome-mediated phototropism underlying a phytochrome-mediated phototropism. If so, then the cryptochrome system in totally etiolated peas is at least 2 orders of magnitude more sensitive than in peas grown under red light (1). Indeed, when plants were pretreated with blue light, both first and second positive phototropisms were entirely suppressed.

Finally, the results of the position transducer experiments reported here are somewhat different from those obtained by Cosgrove (6). When he illuminated Alaska peas from the side with 5 min of strong red light he obtained a transient initial reduction in rate, followed by an overshoot past the preirradiation rate within 20 to 30 min after the start of the irradiation. He saw a more sustained decrease in growth rate beginning 30 to 40 min after the start of the illumination, which contrasts with the more rapid sustained growth decrease reported here. The notable differences between the conditions he used and those in this report are that he used green safelights when handling his plants, and that he irradiated his plants from the side. Bleiss and Smith (3) reported a rapid decrease in extension growth in wheat seedlings in response to a short pulse of red light. They also reported that if they mounted plants onto a position transducer apparatus under dim green safelights, then conducted the rest of the experiment in the dark, the lag period between red light stimulation and a visible change in growth rate increased from about 10 min to about 30 min. It is possible that Cosgrove's plants developed a similar increase in lag period between the red light pulse and a sustained decrease in growth rate as a result of having seen green light.

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