Absence of Pfr Destruction in the Modulation of Phenylalanine Ammonia-Lyase Synthesis of Mustard Cotyledons

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ABSTRACT

The relationship between the amount of active phytochrome (Pfr) produced by 5-minute light pulses and the rate of subsequent enzyme accumulation (phenylalanine ammonia-lyase, EC 4.3.1.5) of mustard (Sinapis alba L.) cotyledons was investigated. The response rapidly adjusts to changes of the Pfr level produced by light pulses of different wavelengths. Regardless of total phytochrome levels in the cotyledons, response adjustments to new photostationary states (ϕPfr) are correlated with ϕPfr values. On the other hand, the kinetics of enzyme accumulation shows no influence of Pfr destruction as determined spectrophotometrically (τPfr = 45 min) in the same organ (see Schäfer et al. 1973 Photosom Photobiol 18: 331-334). It is concluded that the phytochrome molecules involved in the regulation of this response by light pulses comprise a small fraction of the total phytochrome of the cotyledons. In contrast to bulk phytochrome this fraction appears to be not subject to Pfr destruction.

The evidence available to date concerning the quantitative relationship between the amount of active phytochrome [Pfr], and the extent of photomorphogenic responses is complex and in part paradoxical (6, 7, 18). In spite of numerous efforts, the attempts to find a general formula for the quantitative relationship between the effector, Pfr, and the physiological effects produced by its action in the cell have failed. The inability to formulate this relationship in even qualitative terms has been explained by the assumption that there is no general primary action of Pfr but rather a multiplicity of such actions which can differ in their kinetic and mechanistic properties (12). The most impressive discrepancies exist between threshold responses, in which Pfr acts as an on/off switch operating at a distinct threshold level (12-14, 19) and graded responses which show a steady [Pfr] dependency over several orders of magnitude (4).

In general, the level of Pfr in vivo is calculated from spectrophotometric measurements of phytochrome phototransformations and the dark reactions of Pfr. In mustard seedlings where the contribution of the dark reversion of Pfr to Pr is small, destruction of Pfr has been found to be the physiologically relevant dark reaction of the phytochrome system (14). Indeed, in the case of threshold responses, a phytochrome model consisting of Pr synthesis, Pr = Pfr phototransformations, and Pfr destruction is necessary and sufficient to elaborate a quantitative Pfr response relationship which can be used as a physiological assay for Pfr (12). The strict log linearity of this assay between 80 and 1.25% of total phytochrome implies a constant Pfr half-life even at very small Pfr levels. However, this relationship holds true only at Pfr levels above the threshold. To maintain the relationship at lower [Pfr] requires the assumption that destruction of Pfr does not take place below the threshold (13). There are several other reports in the literature suggesting the existence of physiologically stable Pfr also in nonthreshold responses (3, 8, 20, 23).

In the present paper we investigate the question of whether Pfr destruction plays a role in the regulation of the de novo synthesis (21, 22) of PAL, a graded phytochrome response of the mustard seedling cotyledons. The level of this enzyme rapidly responds to the formation and removal of Pfr (2), a prerequisite for the clear physiological demonstration of active Pfr levels.

MATERIALS AND METHODS

The experimental materials, including the batch of mustard (Sinapis alba L.) seeds and germination procedures (25 C) are described elsewhere (1). In addition to the standard red (0.76 w m⁻²) and far red (3.5 w m⁻²) light sources, Leitz Prado projectors with the following Schott interference filters were used: DIL 665 nm (0.7 w m⁻²), DEPIL 699 nm (2 w m⁻²), DIL 705 nm (2 w m⁻²), DEPIL 710 nm (2 w m⁻²), DEPIL 714 nm (2 w m⁻²), AL 714 nm (3.5 w m⁻²), DEPIL 724 nm (1.7 w m⁻²), AL 756 nm (7 w m⁻²). A 5-min irradiation is saturating for establishment of the photostationary states of phytochrome in mustard hypocotyl hooks at all wavelengths used (12).

PAL activity and anthocyanin content of cotyledons were assayed as described (1, 9). Experimental values in figures are based on four to 10 independent determinations (averages of 40 pairs of cotyledons and are presented ± se where appropriate). The unit of enzyme activity is the pkatlas (i.e. pmol trans-cinnamic acid sec⁻¹) per pair of cotyledons.

RESULTS AND DISCUSSION

A straightforward approach to relate the extent of a physiological response to different Pfr levels is to determine the effect of short light pulses producing various amounts of Pfr (e.g. pulses of different wavelengths establishing various photostationary states, ϕPfr). Figure 1 shows this type of "Pfr response curve" for the induction of PAL synthesis in mustard cotyledons. Since the function, ϕ = f(ϕPfr), is not precisely known in this tissue (5) we cannot reliably quantitate the correlations between ϕPfr and the size of the response. However, the data indicate a continuous dependency of induced PAL activity on the amount

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4 Abbreviations: PAL: phenylalanine ammonia-lyase (EC 4.3.1.5); Pr, Pfr: red- and far red-absorbing forms of phytochrome (brackets denote concentrations); ϕPfr = [Pfr]l/[Pr] + [Pfr]: photostationary state of phytochrome.
of Pfr through the whole range of photostationary states between red ($\phi_{665} = 0.8$) and far red ($\phi_{756} = 0.001$) light. Similar relationships have been observed by other workers (11).

The accumulation of PAL ceases instantaneously when Pfr is removed from the tissue (Fig. 2 and ref. 2) indicating a permanent requirement for effector to maintain the increase of enzyme. It is to be expected that a decrease of active Pfr by destruction leads to a concomitant slowing down of the response which should be quantitatively related to the disappearance of Pfr with time. In mustard cotyledons, the spectrophotometrically determined half-life of Pfr (bulk phytochrome) is 45 min at 25 C (17). Thus, if the Pfr involved in PAL induction were subject to this destruction, the rate of the response should decrease within 10 half-lives (8 hr) to a value which corresponds to a Pfr level about $10^{-3}$ times that present at time zero. Fig. 3 shows that this expectation is not met by the kinetics of PAL accu-

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Effect of various photostationary states established by brief saturating irradiations with different wavelengths on the induction of PAL synthesis. Seedlings were irradiated for 5 min with strong monochromatic light (interference filters, cf. "Materials and Methods") at 36 hr after sowing. Enzyme activity was determined 8 hr later (cf. Fig. 3). The curve for the photostationary state $\phi$, is calculated from spectrophotometric and physiological measurements with mustard hypocotyl hooks and maize coleoptiles (16).

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Cessation of PAL increase after removal of Pfr. Enzyme synthesis was induced by continuous standard far red light (high irradiance response). Before the seedlings were transferred to darkness (arrows) [Pfr] was reduced to a low level (cf. Fig. 1) by a saturating pulse (5 min) of 756 nm light.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Kinetics of PAL induction by brief saturating light pulses in the range of 665 to 756 nm. Seedlings were irradiated for 5 min at time zero with the wavelengths indicated. (These experiments are independent of and therefore do not exactly agree with those of Fig. 1.)

- **665 nm**
- **718 nm**
- **756 nm**
- **699 nm**
- **714 nm**
- **724 nm**

**dark control**

The rate of the response is related to the amount of Pfr established by pulses of different wavelengths in a graded manner according to Figure 1, there is no detectable deviation from linearity of the enzyme increase over at least 8 hr. Thus, we may conclude that the spectrophotometrically assayable disappearance of photoreversible Pfr in the cotyledons has no relevance for the mediation of this response. The formation of anthocyanin which is an end product of the biochemical pathway initiated by PAL follows the same quantitative pattern after a time lag of about 4 hr (Fig. 4). Evidently, the rate of anthocyanin synthesis is quantitatively correlated with PAL activity but the kinetic data do not suggest PAL formation as the immediate trigger of anthocyanin synthesis.

Aside from the situation in continuous light (high irradiance reaction, Fig. 2), the response could become insensitive to the Pfr level shortly after a brief irradiation, i.e. the response might proceed in the following dark period not according to the actual but to the previously established Pfr level. To test this possibility, the seedlings were irradiated with a second pulse of red or 756 nm light 1, 2, 3, 4, and 6 hr after the first pulse (21). Figure 5 shows representative results. The system responds to a second pulse in much the same way as to the first one. A 756 nm pulse at 40 hr reverses the effect of a previous red pulse but produces an additional increase if the first pulse was also 756 nm light. Similarly, if both pulses are red light, the second pulse induces a further increase in the rate of PAL formation. Such cumulative effects are generally observed in experiments with two or more light pulses separated by sufficiently long dark periods. In the present case Figure 1 indicates that the "super-induction" by the second pulse cannot be accounted for by the small increase of total phytochrome through Pr synthesis during the 4-hr dark period (about 25% increase based on the level present at 36 hr after sowing; see ref. 17). We have to assume
FIG. 4. Relationship between PAL activity and rate of anthocyanin accumulation. Seedlings were irradiated for 5 min at time zero with standard red, standard far red, and 756 nm light. The first derivative of anthocyanin accumulation was calculated from the accumulation kinetics.

![Graph showing PAL activity and anthocyanin accumulation over time](image)

**Fig. 5.** Effect of a readjustment of the photostationary state by a second light pulse on PAL activity. Seedlings were irradiated for 5 min with standard red or 756 nm light at 36 hr after sowing (first pulse). After 4 hr a second 5-min pulse of either red or 756 nm light was administered to red-, 756 nm-, and dark-pretreated seedlings.

![Graph showing PAL activity with and without a second light pulse](image)

that the sensitivity of the PAL-producing system to Pfr increases with time. A general increase of the response to light treatments during this period of mustard seedling development is a common observation (10). The important feature of the kinetics shown in Figure 5 is that the rate of enzyme increase produced by the second light pulse is the same in preirradiated and in not-preirradiated cotyledons. This markedly contrasts with the different Pfr levels at this point calculated on the basis of a Pfr half-life of 45 min. (The amount of total phytochrome would be about 50 and 125%, respectively, in red preirradiated and not-preirradiated cotyledons, based on the level present at 36 hr after sowing. This ratio corresponds approximately to the ratio of effectiveness between 695 and 660 nm light; see Fig. 1). As a whole, the kinetics of Figure 5 indicates that the system still responds normally to changes of the Pfr level if these changes are produced by a reestablishment of photostationary states. Thus, the response has not yet escaped from control by Pfr during the dark period.

Dependence on Pfr destruction in this system would require the assumption that the continuous loss of Pfr after a light pulse is quantitatively compensated for by a concomitant, Pfr-dependent increase of the responsiveness toward Pfr. On the basis of present knowledge, such a mechanism appears rather unlikely. Thus, the data presented above appear to be easily compatible only with the conclusion that those Pfr molecules which are involved in the mediation of PAL induction by light pulses are not subjected to destruction as determined spectrophotometrically in the cotyledons. It is conceivable that this fraction is only a minor part of the total phytochrome of these organs, especially since the induction of PAL and anthocyanin synthesis by light pulses is largely restricted to the lower epidermis in mustard cotyledons (24). In contrast, the synthesis of PAL and quercetin (+ other yellow flavonoids) mediated by continuous far red light takes place in the upper epidermis (24). Therefore it may not be justified to extrapolate from the light pulse experiments presented in this paper to the high irradiance reaction (15). In other light pulse responses of the mustard seedling, e.g. repression of lipoygenase synthesis and phytochrome growth, the destruction of phytochrome active Pfr with the spectrophotometrically determined half-life of 45 min has unambiguously been demonstrated (12–14, 19). We concluded that different phytochrome responses of a plant can differ qualitatively with respect to the dark reactions of the Pfr involved. Multiple mechanisms of action have already been deduced from previous physiological work with the mustard seedling (12).

**LITERATURE CITED**

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