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MEMBRANE PERMEABILITY CHANGES: A COMPONENT OF PHYTOCHROME-MEDIATED ANTHOCYANIN SYNTHESIS IN *SINAPIS ALBA*

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Key Word Index—*Sinapis alba*; Cruciferae; anthocyanin; phytochrome; membrane permeability.

Abstract—Dark synthesis of anthocyanin in *Sinapis alba* seedlings is greatly promoted by short treatments with *n*-propanol. The effect of *n*-propanol treatment is reversed by subsequent far-red light pulses. Photoreversibility kinetics suggest that *n*-propanol and red light act in the same way. However, phytochrome measurements and other control experiments suggest that *n*-propanol treatment does not lead to significant Pfr production, nor does it increase the effectiveness of any 'dark' Pfr present in the seedlings. The findings are difficult to explain in terms of Pfr as the sole effector of this phytochrome-mediated response.

INTRODUCTION

Attempts to correlate phytochrome-controlled responses quantitatively with the known properties of phytochrome have generally been based upon the assumption that Pfr, the far-red absorbing form of phytochrome, is the active form of phytochrome [1]. It has been assumed that Pfr interacts in some way with a reaction partner, probably an undefined biological membrane, this interaction eventually leading to the display of the response: Pfr + X → PfrX → response. However, the evidence suggests that only a small number of responses (e.g. the activity of lipoxygenase in *Sinapis alba*) exhibit strong correlations with [Pfr] [2] as measured. A lack of correlation is especially apparent under conditions of prolonged irradiation; that is, in those responses that show the typical 'high irradiance reaction' but, even under inductive conditions (short pulses of red and far-red light) a number of a 'non-rational' relationships exist between spectrophotometrically detectable [Pfr] and the extent of the response [3, 4].

Much of the evidence to support the view that Pfr is the effector of phytochrome-mediated responses has been obtained from the study of the photocontrol of anthocyanin synthesis in *S. alba* [5–7]. Phytochrome-mediated anthocyanin synthesis has been used extensively as a 'model' photomorphogenic response. Furthermore Lange *et al.* have concluded that, under inductive conditions, this response is mediated exclusively by Pfr, a view supported by other workers [6, 7]. However, recent results of Johnson [8] have cast doubt on the validity of this conclusion and schemes to account for phytochrome action have been proposed in which importance is placed upon the location of phytochrome in important cellular membranes, where it functions to control the passage of metabolites through the membrane [9]. Pecket and Bassin have demonstrated that increases in membrane permeability (achieved by treatment of the tissue with reagents such as *n*-propanol) will result in increased dark synthesis of anthocyanin in red cabbage [10]. Moreover, they also showed that the effects of such increases in membrane permeability on anthocyanin level are reversible by subsequent far-red irradiation. That is to say, far-red light will reverse an effect even though there has apparently been no Pfr formed by the first treatment. It seemed to us worthwhile examining these remarkable observations in greater detail.

With regard to anthocyanin synthesis in *S. alba* it has been claimed that it is improbable that the action of phytochrome is on the permeability of cellular membranes [7]. We have used a similar approach to that of Pecket and Bassim to test this claim by studying the effects of increased membrane permeability on anthocyanin synthesis in *S. alba* in relation to photocontrol. We report here evidence that changes in

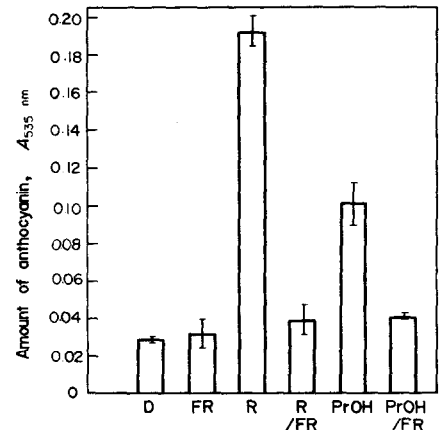

Fig. 1. Effect of R, R/FR, *n*-PrOH and *n*-PrOH/FR on anthocyanin synthesis in *Sinapis alba*. 40-hr-old etiolated seedlings were exposed to 5 min R light or 15 min 0.5 % *n*-PrOH with or without a subsequent 5 min exposure to FR light. Anthocyanin contents were determined at 64 hr after sowing.

membrane permeability are involved in the photoresponse. Data are presented which are difficult to explain if Pfr is the sole effector of this response.

RESULTS AND DISCUSSION

When germinated in darkness on sterile agar, seedlings of *S. alba* accumulate only very small amounts of anthocyanin. Figure 1 shows that exposure of 40-hr-old etiolated seedlings to 5 min of red light promotes a highly significant increase in anthocyanin synthesis, determined after a further 24 hr dark incubation. A 5 min pulse of far-red light (758 nm) when given immediately after a red pulse will completely reverse its effect. Treatment of the seedlings for 15 min with 0.5 % *n*-propanol (*n*-PrOH) also leads to a substantial increase in anthocyanin synthesis during the 24 hr dark incubation. It can also be seen that the effect of the short treatment with *n*-PrOH is reversed by subsequent exposure to 5 min of far-red light. These observations are in general agreement with those of Pecket and Bassim [10] and support their conclusion that an overall increase in membrane permeability results in increased anthocyanin synthesis. Furthermore, since subsequent far-red light will reverse the effect of this presumed increased permeability it can be postulated that the low energy red/far-red reversible reaction for the control of anthocyanin synthesis also involves changes in membrane permeability, i.e. the changes in membrane permeability are under phytochrome control.

Evidence that *n*-PrOH is having the same effect as light is provided by a comparison of the rates of escape from photoreversibility. It was found that with increasing dark periods between red and far-red light pulses there was an escape from photoreversibility until, after 6 hr, a pulse of far-red light no longer reversed the effect of a pulse of red light. It was also found that after this time the effect of *n*-PrOH treatment could no longer be reversed by subsequent far-red light. If the data for escape from photoreversibility are compared (Fig. 2), it can be seen that red light-treated and *n*-PrOH-treated material escape from photoreversibility at similar rates, the time for a 50 % loss being between 2 and 3 hr. This similarity in escape rate is a strong indication that *n*-PrOH and red light are having the same effect, increasing membrane permeability.

A number of alternative explanations for these findings exist, namely, that *n*-PrOH treatment itself leads to the formation of Pfr, that *n*-PrOH treatment enhances the rate of photoconversion of Pr → Pfr such that significant amounts of Pfr are produced by the green 'safelight', or that *n*-PrOH treatment enhances the effect of any 'dark' Pfr that may be present in the seedlings. We have attempted to account for these trivial explanations in a series of control experiments (Table 1). First, a 15 min treatment with *n*-PrOH in complete darkness produces a response which is not significantly different from the response observed when the manipulations are performed under green 'safelight'. It seems unlikely, therefore, that the response is a consequence of Pfr formed by the safelight. Secondly, the removal of any 'dark' Pfr by a 10 min pulse of 758 nm far-red light, which produces <0.1 % Pfr [2], at 15 hr after sowing does not significantly alter the response, indicating that the effect of *n*-PrOH is not a consequence of the enhancement of the effectiveness of 'dark' Pfr. Finally spectrophotometric analysis of *n*-PrOH-treated and untreated seedlings revealed no significant differences in total phytochrome nor evidence of any Pfr within the limits of detection of the spectrophotometer (Whitelam, G. C., unpublished). The evidence, therefore, supports the view that changes in membrane permeability, occurring either as a result of the absorption of light by phytochrome or promoted directly by *n*-PrOH, function in the control of anthocyanin synthesis.

A difficulty arises in explaining the far-red reversibility of the *n*-PrOH-promoted response. It was seen in Fig. 1 that a 5 min pulse of far-red light, which itself produces some Pfr, will nevertheless reverse the effect of *n*-PrOH treatment. This is most unexpected since *n*-PrOH

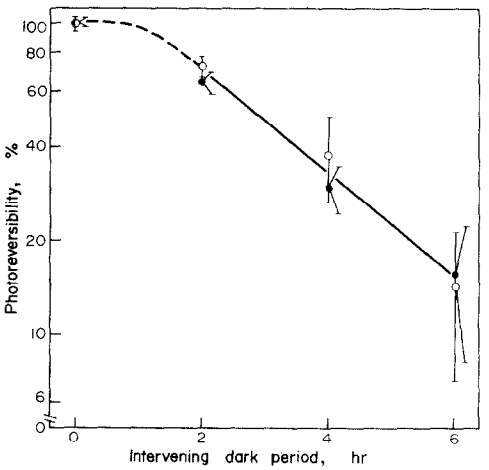

Fig. 2. Escape from photoreversibility. Effect of increasing dark periods between either a 5 min pulse of R light (○) or a 15 min exposure to 0.5 % *n*-PrOH (●) and a 5 min pulse of FR light on anthocyanin synthesis in 40-hr-old *Sinapis alba*. Anthocyanin contents were determined at 64 hr after sowing.

Table 1. Effect of *n*-PrOH on anthocyanin synthesis in *Sinapis alba* as influenced by (a) the green safelight and (b) the removal of dark Pfr.

Treatment	Amount of anthocyanin *A* at 535 nm
(a)	
15 min H_2O dark	0.026 ± 0.004
15 min H_2O green safelight	0.030 ± 0.005
15 min *n*-PrOH dark	0.091 ± 0.007
15 min *n*-PrOH green safelight	0.089 ± 0.009
(b)	
15 hr dark + 10 min 758 nm + 25 hr dark + 15 min H_2O	0.025 ± 0.001
15 hr dark + 10 min 758 nm + 25 hr dark + 15 min *n*-PrOH	0.095 ± 0.010

(a) 40-hr-old etiolated seedlings were treated with 0.5 % *n*-PrOH either in total darkness or under green safelight.

(b) Following the removal of 'dark' Pfr at 15 hr from sowing (10 min, 758 nm) the effect of *n*-PrOH treatment at 40 hr was observed. In all cases anthocyanin contents were determined at 64 hr from sowing. Standard errors are shown.

treatment does not produce detectable Pfr. Clearly this result cannot be explained in terms of anthocyanin synthesis, being dependent exclusively on the level of Pfr. This conclusion was confirmed using an approach similar to that employed by Mohr *et al.* [11]. They exploited the use of light pre-treatments, given prior to 'competence' for Pfr, on subsequent phytochrome control of anthocyanin synthesis in *Sinapis alba*. It had been found that red light pre-treatments prior to 24 hr from sowing could be fully reversed by far-red light pulses at 24 hr. Nevertheless, these early red light pulses increased the subsequent effectiveness of red light pulses given at or after 24 hr from sowing. Thus the early pulses, whilst having no effect on their own, enhanced the effectiveness of subsequent pulses. We wanted to test whether *n*-PrOH would substitute for light pulses given prior to competence (i.e. before 24 hr). The results are presented in Table 2. The effectiveness of a red light pulse at 24 hr from sowing is decreased by a far-red light pulse at 12 hr from sowing (a result confirming the presence of seed Pfr). Taking this into account, a red light pulse at 12 hr from sowing enhances the effect of a red light pulse at 24 hr. This enhancement is reversed if the red pulse at 12 hr is followed by a far-red light pulse. These results are in agreement with the data of Mohr *et al.* [11]. However, a 15 min treatment with *n*-PrOH given at 12 hr also enhances the effectiveness of the subsequent red light pulse and its effect is also far-red reversible. Again this result confirms that far-red light will reverse the effect of a treatment which has not itself yielded Pfr. Furthermore in this instance the effect is observed before the tissue is competent to respond to Pfr.

In order to account for these observations it seems to us that there must be a second component to phytochrome action other than the presence of Pfr. Furthermore this second component can be simulated by *n*-PrOH, but must also occur upon irradiation. We suggest as a working hypothesis that irradiation actively facilitates the change in membrane permeability but the final permeability depends upon the ultimate level of Pfr. It has been proposed elsewhere that phytochrome responses can be explained by assuming that a phytochrome cycling-generated reaction partner for Pfr is one of two essential effectors of phytochrome action [12]. The present data provide physiological evidence for the involvement of a second component to phytochrome action, a component most likely associated with membrane permeability changes.

Table 2. Influence of pre-treatments, prior to competence, on subsequent phytochrome-controlled anthocyanin synthesis in *Sinapis alba*

Treatment	Amount of anthocyanin *A* at 535 nm
Dark	0.030 ± 0.001
24 hr dark + 5 min red	0.073 ± 0.003
12 hr dark + 10 min 758 nm + 12 hr dark + 5 min red	0.056 ± 0.003
12 hr dark + 5 min red + 10 min 758 nm + 12 hr dark + 5 min red	0.046 ± 0.002
12 hr dark + 15 min *n*-PrOH + 10 min 758 nm + 12 hr dark + 5 min red	0.052 ± 0.001
12 hr dark + 5 min red + 12 hr dark + 5 min red	0.068 ± 0.002
12 hr dark + 15 min *n*-PrOH + 12 hr dark + 5 min red	0.080 ± 0.003

Etiolated seedlings were treated as indicated and anthocyanin contents were determined at 64 hr from sowing. Standard errors are shown.

EXPERIMENTAL

Plant material and growth conditions. Seeds of *Sinapis alba* (Asgrow, Freiburg-Ebnet, 1972 harvest, kindly supplied by Professor H. Mohr) were germinated in Petri dishes on sterile 1% aq. agar, at 25°.

Light sources. The red light source consisted of a bank of 'Grolux' fluorescent tubes filtered through one layer of No. 1 and one layer of No. 6 Cinemoid (Rank Strand Electric). The photon fluence rate at seedling height was $\approx 7\,\mu mol\,m^{-2}s^{-1}$. Far-red light was provided by a Schott interference filter, type AL of $t\lambda$ max 758 nm. All irradiations were performed at 25°. The safelight consisted of a 6 W Mazda daylight fluorescent tube, covered by 6 layers of No. 39 (Primary green) Cinemoid. The fluence rate was less than $0.01\,\mu mol\,m^{-2}s^{-1}$.

n-Propanol treatments. Short-duration (15 min) exposure of seedlings was achieved by transfer of the seedlings from the original dish to a fresh Petri dish containing two Whatman No. 1 filter papers moistened with 4 ml of 0.5% *n*-PrOH, followed by transfer back to the original dish. As a control, all other seedlings were routinely transferred to a dish containing filter papers moistened with distilled water. Although it was noted that prolonged exposure to *n*-PrOH resulted in an inhibition of root development, these manipulations and the short *n*-PrOH treatments resulted in no noticeable damage to the seedlings.

Anthocyanin extraction and assay. Anthocyanin from samples of 15 seedlings was extracted and assayed following the method of Lange *et al.* [5].

REFERENCES

1. Schäfer, E. (1975) *J. Math. Biol.* **2,** 41.
2. Oelze-Karow, H. and Mohr, H. (1973) *Photochem. Photobiol.* **18,** 319.
3. Briggs, W. R. and Chon, H. P. (1966) *Plant Physiol.* **41,** 1159.
4. Hillman, W. S. (1966) *Plant Physiol.* **41,** 907.
5. Lange, H., Shropshire, W. Jr. and Mohr, H. (1971) *Plant Physiol.* **47,** 649.
6. Steinitz, B., Schäfer, E., Drumm, H. and Mohr, H. (1979) *Plant, Cell Environ.* **2,** 159.
7. Drumm, H. and Mohr, H. (1974) *Photochem. Photobiol.* **20,** 151.
8. Johnson, C. B. (1980) *Plant, Cell Environ.* **3,** 45.
9. Smith, H. (1975) *Phytochrome and Photomorphogenesis.* McGraw-Hill, London.
10. Pecket, R. C. and Bassim, H. T. A. (1974) *Phytochemistry* **13,** 815.
11. Mohr, H., Drumm, H., Schmidt, R. and Steinitz, B. (1979) *Planta* **146,** 369.
12. Johnson, C. B. and Tasker, R. (1979) *Plant, Cell Environ.* **2,** 259.