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THE EFFECT OF MEMBRANE STABILIZERS ON PHYTOCHROME-CONTROLLED ANTHOCYANIN BIOSYNTHESIS IN *BRASSICA OLERACEA*

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Key Word Index—*Brassica oleracea*; Cruciferae; red cabbage; anthocyanin; phytochrome; membrane permeability; membrane stabilizers.

Abstract—The promotion of anthocyanin synthesis in red-cabbage seedlings by 5 min exposure to R light is inhibited by subsequent application of $CaCl_2$. The stimulation of dark synthesis of anthocyanin by *n*-PrOH and by kinetin is also reduced by Ca^{2+} and by cholesterol, both of which are well known to stabilize cell membranes. By contrast, EDTA, which chelates Ca^{2+}, promotes dark synthesis of anthocyanin. Assay of native Ca^{2+} extractable from seedlings immersed in EDTA demonstrates that R light exposure promotes a highly significant increase in extractable Ca^{2+}. It is suggested that the molecular configuration of the phytochrome molecule affects the ability of a membrane to bind Ca^{2+} and that this in turn affects the permeability to substrates which are required for anthocyanin biosynthesis.

INTRODUCTION

It has been suggested previously from work on the low-energy red/far-red (R/FR) reversible control of anthocyanin biosynthesis that the locus of phytochrome action is in a membrane [1]. Evidence for this view came from the fact that reagents such as *n*-propanol (PrOH), which are believed to increase membrane permeability, cause a stimulation of dark synthesis of anthocyanin in red cabbage which can be nullified by exposure to far-red (FR) light [1]. It was suggested that the molecular configuration of phytochrome controls the passage of a substrate(s) through a membrane to the site of anthocyanin biosynthesis. Further evidence for this view was provided by the fact that exogenously applied phenolic precursors, such as shikimic acid, promote dark synthesis of anthocyanin much more markedly after treatment of the tissue with PrOH. Evidence has also been presented that kinetin, like red light, promotes anthocyanin synthesis in dark-grown red cabbage and that its effect can be reversed by FR light [2].

If the effects of light, kinetin and reagents such as PrOH are indeed upon membrane permeability, then it would be expected that reversals of their effects should be brought about by the application of reagents such as calcium chloride [3, 4] and cholesterol [5], which are well known to act as membrane stabilizers. The work reported here concerns the further investigation of the membrane based phytochrome control of anthocyanin synthesis as evidenced by the effects of these membrane stabilizing agents.

RESULTS AND DISCUSSION

In preliminary work it was established that the biosynthesis of anthocyanin in dark-grown seedlings of red cabbage is unaffected by treatment with $CaCl_2$ over the range 0–10 mg/l. However, the application of $CaCl_2$ to 2-day-old dark-grown seedlings following a 5 min exposure to R light nullifies the effect of the light treatment (Table 1). These results are consistent with the view that Ca^{2+} has a stabilizing effect on a membrane which

Table 1. Effect of $CaCl_2$ and cholesterol on anthocyanin synthesis in *Brassica oleracea*

Treatment*	Anthocyanin content (A 525 nm)† Dark	R	PrOH	Kinetin
Water	0·34	0·41	0·59	0·45
$CaCl_2$	0·32	0·33	0·40	0·33
Cholesterol	0·37	0·37	0·37	0·39

* Seedlings were grown for 2 days in darkness and then treated for 5 min with R light, or with 1% PrOH, or 0·2% kinetin, for 15 min. Immediately after these treatments $CaCl_2$ (7 mM) or cholesterol (0·1 mM) was applied to the seedlings for a further period of 48 hr in darkness.

† For 10 seedlings extracted in 10 ml 1% HCl in 1 cm cell.

is involved in the regulation of the passage of a substrate(s) of anthocyanin biosynthesis to the enzymes involved in the synthesis [1].

The stimulation of dark synthesis by PrOH and by kinetin is also markedly reduced by subsequent treatment with $CaCl_2$ (Table 1). Furthermore, cholesterol is similarly capable of reducing the stimulatory effects of R light, PrOH and kinetin (Table 1). An alcohol promoted increase in membrane permeability as evidenced by betacyanin efflux from beetroot, which is inhibited by Ca^{2+}, has been reported previously [6].

If the effect of exogenously applied Ca^{2+} is to stabilize the membrane and thereby prevent the increase in permeability induced by such factors as R light, then it is possible that native Ca^{2+} in the membrane may itself be involved in the normal regulation of the passage of substrates. In order to explore this possibility, the influence upon anthocyanin synthesis of ethylenediaminetetraacetic acid (EDTA), which chelates Ca^{2+} [7, 8], was investigated. Seedlings were treated with EDTA for

Table 2. Effect of EDTA on phytochrome-controlled anthocyanin synthesis in *Brassica oleracea*

Treatment*	Anthocyanin content (A 525 nm)† Dark	R	FR
Water	0·36 ± 0·02	0·47 ± 0·02	0·36 ± 0·03
EDTA before light	0·44 ± 0·04	0·46 ± 0·04	0·45 ± 0·05
EDTA after light	0·44 ± 0·04	0·49 ± 0·02	0·35 ± 0·03

* Seedlings were grown for 2 days in darkness and then treated with either EDTA (0·5 mM) or water for 15 min before or after exposure to R or FR. Period of dark incubation subsequent to treatment was 48 hr. The figures are means of 8 observations and standard errors are shown.

† See Table 1.

Table 3. Influence of R, FR and darkness on extractable Ca^{2+} from seedlings of *Brassica oleracea*

Treatment*	Extractable Ca^{2+} (ppm) Dark	R	FR
Water	3·7	4·1	3·4
EDTA	13·4	17·0	12·5

* Seedlings were grown for 2 days in darkness and then exposed to R or FR for 5 min followed by immersion in water or EDTA (0·5 mM) for 15 min, after which time the seedlings were removed and the extracted Ca^{2+} content of the solution was determined. The figures are means of five observations. The difference between R + EDTA and EDTA is highly significant and the variance ratio = 59·58. Least significant difference (1% level) between treatment means = 3·11.

15 min before or after 5 min exposure to R or FR light. EDTA itself stimulates anthocyanin synthesis in dark-grown red cabbage seedlings (Table 2). When applied either before or after a R light treatment EDTA does not appear to promote a greater stimulation than R light on its own. However, when a FR exposure is employed instead of R, the effect depends upon the sequence of the treatments. Application of EDTA promotes synthesis when applied before the FR exposure but not when the sequence is reversed (Table 2). These results suggest that FR light may in some way render native Ca^{2+} less readily chelatable by EDTA but that if the EDTA treatment precedes the FR exposure, insufficient free Ca^{2+} are present to stabilize the membrane.

Anthocyanin synthesis in red cabbage seedlings takes place in the epidermal and sub-epidermal cells of the cotyledons and hypocotyl. The principal site of synthesis is the cotyledons and it would therefore appear likely that the effects of EDTA reported above arise following the uptake and translocation of this substance to the sites of anthocyanin synthesis. In an attempt to ascertain whether the above explanation concerning the binding of Ca^{2+} might be valid, assays of extractable Ca^{2+} were carried out on solutions in which seedlings had been completely immersed for 15 min after a 5 min exposure to R or FR light. When water was used for extraction, only small quantities of Ca^{2+} were removed and no significant differences were found between the treatments (Table 3). However, when EDTA was employed as extractant, a substantially larger amount of Ca^{2+} was removed and there was a highly significant further increase in extractable Ca^{2+} from seedlings which

had received a prior exposure to 5 min R light (Table 3). Assays of extractable Ca^{2+} from separated cotyledons and hypocotyls revealed that approximately twice as much Ca^{2+} came from the former organs, the principal site of anthocyanin synthesis.

The above observations suggest that the photocontrol of anthocyanin biosynthesis may involve changes in permeability which result from alterations in the capacity to bind Ca^{2+} into a membrane. It seems possible that the extent to which Ca^{2+} are bound into and thereby stabilize the membrane, is a function of the molecular configuration of the phytochrome molecule. If this is so, then the Pr form of phytochrome must have a greater capacity to bind Ca^{2+} than the Pfr form. It is generally assumed that Pfr is the active form of phytochrome in respect of the promotion of photomorphogenetic events [9]. The interpretation placed upon the present data implies that the activity of Pfr may result from it being less able to bind Ca^{2+} in a membrane(s). The increase in permeability which would result would lead to the freer movement through the membrane(s) of substances of importance in plant development. The evidence presented here is consistent with Pr exercising a restraint upon the movement of substrates for anthocyanin biosynthesis by virtue of its greater ability to bind Ca^{2+}.

EXPERIMENTAL

Plant materials and growth conditions. Seeds of red cabbage (*Brassica oleracea* L. cv. Stockley's Giant Red) were obtained from Hurst, Gunson, Cooper and Taber Ltd. The growing conditions, light sources and treatments were as previously reported [1].

Anthocyanin extraction and assay. As reported previously [1]. The absorbance values quoted are means for samples of 10 seedlings extracted in 10 ml of 1% HCl, examined in a 1 cm cell.

Propanol, kinetin and EDTA treatments. Method as reported previously [1, 2]. Seedlings were transferred in a dark room to a fresh Petri dish containing 4 ml of soln for 15 min followed by return to the original dish. Control seedlings were treated in the same way using H_2O. A similar procedure was adopted for the $CaCl_2$ and cholesterol treatments but the seedlings were kept in the reagents for 48 hr.

Extraction and assay of calcium. 80 Seedlings were immersed in 20 ml EDTA (0·5 mM) or H_2O for 15 min. The eluate was taken to dryness and the residue dissolved in 20 ml H_2O. To 1 ml an equal vol. of 6·5% lanthanum was added and the Ca^{2+} content assayed with an atomic absorption spectrophotometer.

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REFERENCES

1. Pecket, R. C. and Hathout Bassim, T. A. (1974) *Phytochemistry* **13,** 815.
2. Pecket, R. C. and Hathout Bassim, T. A. (1974) *Phytochemistry* **13,** 1395.
3. Davson, H. (1951) *Textbook of General Physiology*, J. and A. Churchill Ltd., London.
4. Burström, H. (1952) *Physiol. Plant.* **5,** 391.
5. Grunwald, C. (1968) *Plant Physiol.* **43,** 484.
6. Siegel, S. M. and Halpern, L. A. (1964) *Proc. Nat. Acad. Sci. U.S.* **51,** 765.
7. Burström, H. (1968) *Biol. Rev.* **43,** 287.
8. Foote, B. D. and Hanson, J. B. (1964) *Plant Physiol.* **39,** 450.
9. Hendricks, S. B. and Borthwick, H. A. (1967) *Proc. Nat. Acad. Sci. U.S.* **58,** 2125.