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On the Mechanism of the Changes in Phenylalanine Ammonia-lyase Activity Induced by Ultraviolet and Blue Light in Gherkin Hypocotyls[1]

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ABSTRACT

Irradiation with ultraviolet light causes in the hypocotyl of dark-grown gherkin seedlings the partial conversion of *trans*-hydroxycinnamic acids to the *cis*-isomers. The *trans*-hydroxycinnamic acids inhibit the development of phenylalanine ammonia-lyase activity, and the transformation of these compounds to the much less inhibitory *cis*-isomers forms a ready explanation for the increase in phenylalanine ammonia-lyase activity in the hypocotyl of gherkin seedlings irradiated with ultraviolet light. Arguments are advanced that the increase in phenylalanine ammonia-lyase activity caused by irradiation with blue light is also (at least in part) initiated by *trans-cis* isomerisation of the hydroxycinnamic acids.

Since Zucker's paper (31) on the induction of PAL[2] (EC 4.3.1.5) by light in potato tuber tissue the photoinduction of this enzyme has been studied in a great number of other plants, but the mechanism of this process has so far remained obscure (4, 22, 27, 32). Previously (6), I postulated that photoactivation of one or more photoreceptors starts a series of dark reactions that lead to the formation of a factor which promotes PAL synthesis. This concept will probably have to be revised. In more recent investigations (9–14), evidence has been obtained that the PAL level in gherkin hypocotyls is controlled by end products of the shikimic acid pathway: cinnamic acid, *p*-coumaric, and ferulic acid, and/or glucose esters of the latter two compounds. It appeared that increases in PAL activity similar to those brought about by irradiation were obtained with treatments which caused the release of the cinnamic acid derivatives from the tissue (9) or their conversion into other compounds (13, 14). This has led us to the idea that the effect of light on PAL might be due to a temporary diminution in the concentration of (hydroxy)-cinnamic acid(s) in certain cell compartments. The only direct effect of light on these compounds is the *trans-cis* conversion by the UV part of the spectrum (21). Blue light causes a similar reaction, provided that a suitable photoreceptor is present (25). In this paper, the question will be discussed of whether these reactions play a role in the induction of PAL activity in gherkin hypocotyls.

MATERIALS AND METHODS

The experiments were performed with 3-day-old dark-grown gherkin seedlings, *Cucumus sativus* L. cv. "Venlose niet plekkers," strain Tercken VI (15). The irradiations were carried out at 25 C with UV light of 360 μw/cm^2 (Philips HPW, 125 w, λ_{max} 365 nm) and with blue light of 150 μw/cm^2 (6). *In vivo* spectrophotometry of gherkin hypocotyls was carried out in a Cary spectrophotometer with a scattered transmission attachment. In all experiments, one seedling only was used, with paper tissue in the reference beam. The roots were first removed from the seedling, and then it was placed in a special holder in such a way that only light that has passed through the upper part of the hypocotyl reached the photomultiplier. The top of the cotyledons and the lower part of the hypocotyl were surrounded by moist cotton wool. The preparation and maintenance of the aqueous extracts from the gherkin hypocotyls and the assay of PAL were as described in previous publications (6, 15). The separation of the *trans*- and *cis*-isomers of *p*-coumaric and ferulic acid was achieved by two-dimensional chromatography on Whatman No. 1 paper with isopropanol-ammonia-water (10:10:1), followed by 1% acetic acid in water (5, 15). The sugar esters of the hydroxycinnamic acids were hydrolyzed with NaOH (15) before the separation.

RESULTS

Effect of UV Light on the Hydroxycinnamic Acids in Gherkin Hypocotyls. The gherkin hypocotyls contain glucose esters of *p*-coumaric and ferulic acid which have absorption maxima around 310 nm (15). The shoulder at this wavelength in the absorption spectrum of an aqueous extract of the hypocotyls (Fig. 1A) is caused by the presence of these hydroxycinnamic esters. Figure 1B shows that the *in vivo* spectrum of the upper part of the hypocotyl of a dark-grown gherkin seedling has a similar shoulder at 310 nm. Irradiation of such a seedling for 15 min with UV light causes a drop in the absorption at 310 nm, as is the case when an aqueous extract of hypocotyls or a solution of *trans-p*-coumaric acid in water (Fig. 1C) is irradiated for 15 min with the same light. These changes in the absorption spectra apparently have to be attributed to the partial conversion of *trans*-hydroxycinnamic esters to the *cis*-isomers which absorb at shorter wave-

[1] Dedicated to the memory of Milton Zucker who in 1965 was the first to report the photoinduction of phenylalanine ammonia-lyase. The present author was privileged to have many discussions with him in the years thereafter about the regulatory mechanisms concerning this enzyme.

[2] Abbreviation: PAL: phenylalanine ammonia-lyase.

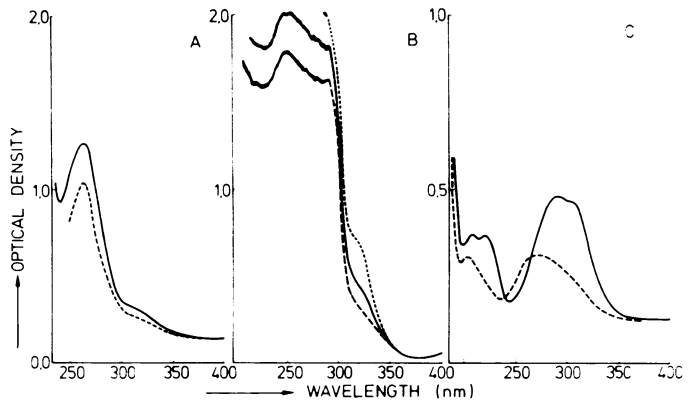

FIG. 1. Changes in the absorption spectra caused by 15 min of irradiation with UV light at 25 C of (A) an aqueous extract of the hypocotyls of dark-grown gherkin seedlings, (B) the upper part of the hypocotyl of a dark-grown seedling, and (C) a solution of *trans-p*-coumaric acid (10^{-5} M) in water. Spectra before the irradiation (——); spectra immediately after the irradiation (– – –); spectrum after 7 hr from the beginning of the irradiation (· · ·).

lengths. By means of two-dimensional paper chromatography it was demonstrated that, prior to the UV irradiation, only *trans*-isomers of esters of *p*-coumaric and ferulic acid had been present in the gherkin hypocotyls and that the irradiation had converted about 40% of each compound to the corresponding *cis*-isomer. Figure 1B shows also that the absorbance at 310 nm increases again if the seedlings after the irradiation are left in darkness. This is due to renewed accumulation of *trans*-hydroxycinnamic esters, as confirmed by paper chromatography.

The technique of *in vivo* spectrophotometry enables us to study the time course of the accumulation of hydroxycinnamic acids by continuous recording of the absorbance at 310 nm. This is shown in Figure 2 for the upper parts of the hypocotyls of two dark-grown seedlings, of which one immediately before being placed in the spectrophotometer was irradiated for 15 min with UV light. In the hypocotyl of the irradiated seedling, the absorbance began to increase after about 25 min, whereas in the dark control no change in absorbance was observed. In some other experiments, there was a small increase in the dark controls, apparently caused by the measuring beam, but this was always less than in the pretreated seedlings. With this method of determining the length of the lag phase for phenol synthesis, with 10 seedlings which had been irradiated for 15 min with UV light, we found an average value of 46 min, measured from the beginning of the irradiation.

Effect of *cis*- and *trans-p*-Coumaric Acid on the PAL Activity *in Vitro* and on the Developent of PAL in Hypocotyl Segments. In a number of studies (3, 16, 23, 24, 28, 29), it had been found that *trans-p*-coumaric acid inhibited the PAL activity. The results presented in Table I show that this compound has a similar effect on the enzyme of the gherkin hypocotyl. It was found that the inhibition was less when the *p*-coumaric acid solution had first been irradiated with UV light until equilibrium had been obtained between *trans*- and *cis*-isomers [about 80% *cis* (21)].

Previously (9), it had been shown that the PAL activity increased in gherkin hypocotyl segments when they were floated on water and that this increase was much less when the water was replaced by an aqueous solution of *trans-p*-coumaric acid. The data in Table II show that inhibition is less if the major part of the *trans-p*-coumaric acid has been converted to the *cis*-isomer before the segments are floated on the solution. By means of paper chromatography, it can be demonstrated that both *cis*- and *trans-p*-coumaric acid accumulate in the segments.

Effect of UV Irradiation on the Development of PAL in Gherkin Hypocotyls. In pea pods (18) and *Petroselinum* cell suspension cultures (30), it has been found that UV light leads to an increase in PAL activity. In Figure 3 the effects of continuous irradiation with our UV source on the PAL level in gherkin hypocotyls are compared with the effects of irradiation with a saturating intensity of blue light. It appears that the length of the lag phase and the time required to reach maximum PAL activity are the same with either light quality. The height of the maximum in PAL activity obtained by irradiation with UV is slightly lower than that resulting from blue

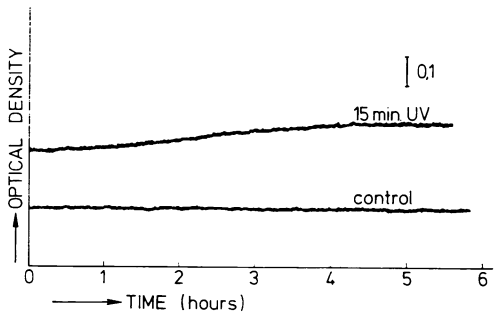

FIG. 2. Continuous recording as a function of time of absorbance at 310 nm in the upper part of the hypocotyl of two dark-grown gherkin seedlings, of which one seedling, immediately before the onset of the recording, had been irradiated with UV light for 15 min.

Table I. *Effect of trans-cis Isomerisation of p-Coumaric Acid on the Activity in Vitro of PAL from Gherkin Hypocotyl Segments*

Solutions containing *cis-p*-coumaric acid were obtained by irradiation of solutions of *trans-p*-coumaric acid with UV light until the equilibrium between the isomers had been obtained. The segments were 2 mm.

Concn *p*-Coumaric Acid	Isomer	
	100% *trans*	20% *trans*, 80% *cis*
M	*PAL activity in % of control*	
10^{-6}	90	97
10^{-5}	76	92
10^{-4}	43	71

Table II. *Effect of trans-cis Isomerisation of trans-p-Coumaric Acid on the Development of PAL activity in Gherkin Hypocotyl Segments Floated on Water*

Solutions containing *cis-p*-coumaric acid were obtained as mentioned in Table I.

Concn *p*-Coumaric Acid	Incubation Time	Isomer	
		100% *trans*	20% *trans*, 80% *cis*
M	*hr*	*PAL activity in % of control*	
10^{-5}	6	38	66
2.10^{-4}	16	39	49
5.10^{-4}	24	17	30

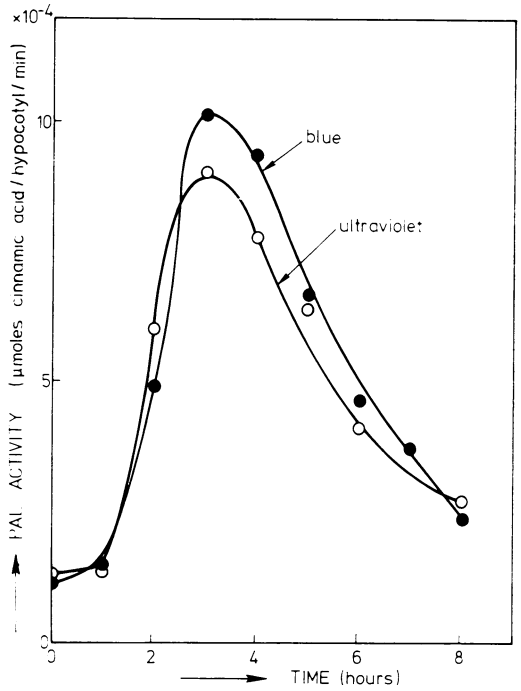

FIG. 3. Time course of the changes in the PAL level in the hypocotyl of dark-grown gherkin seedlings that were continuously irradiated from the time zero with blue or UV light at 25 C.

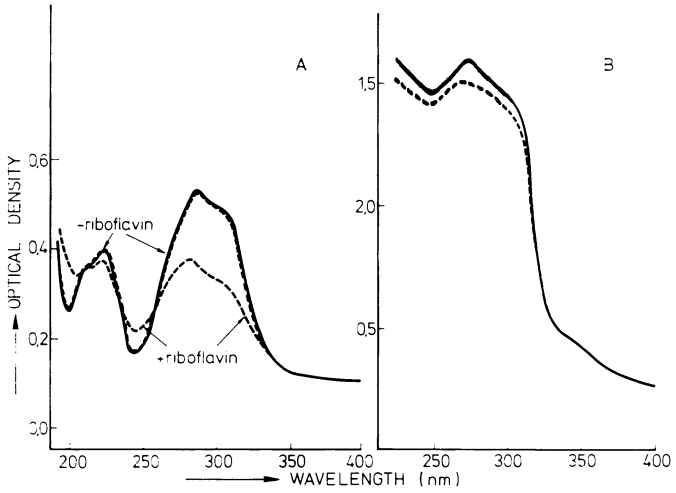

FIG. 4. Effect of irradiation for 15 min with blue light on the absorption spectra of (A) a solution of *trans-p*-coumaric acid in water, with and without riboflavin and (B) the upper part of the hypococtyl of a dark-grown gherkin seedling. The *p*-coumaric acid (10^{-5} M) solution containing riboflavin (2.10^{-6} M) was measured against a solution of riboflavin in water at the same concentration. Spectra before the irradiation (——); spectra after the irradiations (– – –).

light (6) but much higher than that found with saturating red or far red light (7).

Is the Effect of Blue Light on the PAL Level Caused by the Same Mechanism as that of UV Light? The *trans-cis* conversion of *p*-coumaric acid occurs in blue light only if a suitable photoreceptor, *e.g.* riboflavin, has been added to the solution (Fig. 4A). In this experiment, the *p*-coumaric acid solution containing riboflavin was measured against a solution of riboflavin at the same concentration. The spectrum thus obtained was the same as that of the aqueous *p*-coumaric acid solution measured against water, indicating that riboflavin did not interfere with the absorbance spectrum. In view of the fact that photoisomerization of *p*-coumaric acid occurs only in the presence of a photoreceptor, it is not surprising that in the hypocotyl of a gherkin seedling irradiated with blue light no significant drop in the absorption in the 310 nm region (Fig. 4B) is observed, in contrast to the finding in plants treated with UV. The major part of the hydroxycinnamic acids is stored in the vacuoles, and apparently no photoreceptor is present there to sensitize the photoconversion. It might be expected, however, that in those compartments of the protoplasm where both riboflavin and (hydroxy)cinnamic acid(s) are present, photoconversion by blue light of the latter compounds should occur. A slight but consistently observed shift to lower wavelengths of the 250-nm peak in seedlings irradiated with blue light may be an indication of such a reaction. Further evidence in this direction stems from the finding that, after irradiation of dark-grown gherkin seedlings for 30 min with blue light, about 5% of the *p*-coumaric acid in the hypocotyl occurs in the *cis* form. Our chromatographic technique did not reveal this isomer in the dark-grown seedlings or in seedlings irradiated with red light.

DISCUSSION

Gherkin hypocotyls contain, in addition to active PAL, a pool of inactive enzyme (1, 11, 12). Increases in the amount of active enzyme have been obtained by such divergent treatments as (*a*) slicing segments and floating these on water (9), (*b*) treating gherkin seedlings with divalent manganese salts (13), (*c*) spraying them with the herbicide dichlobenil (14), and (*d*) irradiation of the seedlings with UV light. It can be construed that these treatments have in common that they cause a temporary diminution in the concentration of *trans*-(hydroxy)cinnamic acid(s) in certain cell compartments (Fig. 5). There is ample evidence that the (hydroxy)cinnamic acids have a controlling effect on the PAL level (9–11), *viz* increases in their concentration leading to lower enzyme levels and, vice versa, decreases in their concentration leading to higher enzyme levels. It was found that the increases in PAL activity induced by the treatments mentioned above were prevented (partially) by the application of cycloheximide. This may be taken to indicate that the increases in enzyme level result from direct *de novo* synthesis. However, considering the complications arising from the application of cycloheximide to gherkin

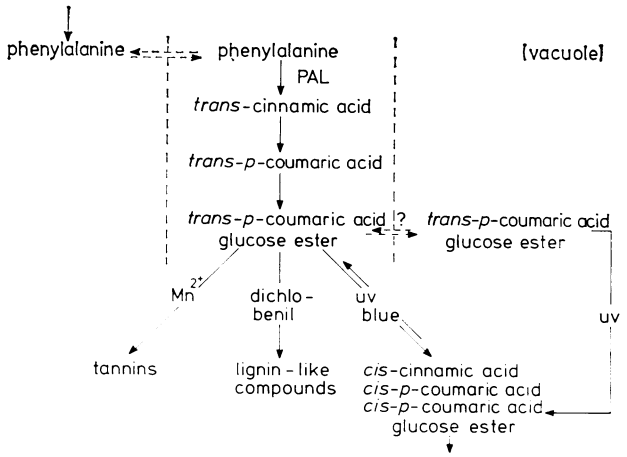

FIG. 5. Scheme to explain the increase in PAL activity in gherkin hypocotyls by various treatments.

seedlings (1, 9, 12), the alternative possibility of activation of pre-existing enzyme cannot be entirely excluded.

The question now arises of whether the effects of light in the visible region can be explained by the same mechanism. It has been stated before (7) that the effect on PAL of a saturating dose of blue light is greater than that of saturating red or far red light. Therefore, a distinction has to be made between the effect of red and far red light, which is probably mediated by phytochrome (2, 26, 27), and that of blue light which must be mediated by another pigment (6–8) (in addition to phytochrome which absorbs also in the blue region). From our experiments it follows that the effect of blue light, at least as far as it is not mediated by phytochrome, might be explained by *trans-cis*-conversion of (hydroxy)cinnamic acid(s) in those cell compartments where a suitable photoreceptor is present. The action spectrum (8) is in agreement with the concept of a flavin type of photoreceptor.

As to the effects of phytochrome, it is generally believed that this photoreceptor affects membrane properties (2, 26). It has been reported (17, 19, 20) that the transport of different cell metabolites is enhanced after conversion of phytochrome to the active form. A possible explanation of the effects of red and far red light on the PAL level is that they cause a phytochrome-mediated increase in the rate of transport of the hydroxycinnamic acids from the site of synthesis to the vacuoles, but evidence for this is not yet available.

Several investigations (3, 16, 23, 24, 28, 29) lead to the conclusion that the (hydroxy)cinnamic acid(s) in addition to affecting the level of active PAL, have also a more direct effect on the enzymatic activity, probably by end product inhibition. The changes that occur after dark-grown gherkin seedlings are exposed to the light can now be explained by the following mechanism. Initially the irradiation causes a decrease in the concentration of *trans*-(hydroxy)cinnamic acid(s) in certain cell compartments. This has a 2-fold effect: it directly enhances the activity of existing PAL and it indirectly causes the formation of more active PAL. Thus it can be understood that (*a*) the time lag for the increase in hydroxycinnamic acids (Fig. 2) is shorter than the time lag for the increase in the PAL level (Fig. 3) and that (*b*) under certain conditions irradiation causes an increase in hydroxycinnamic acid synthesis without causing changes in the PAL level, *viz.* after a short irradiation with red light (7). In the next phase there is a growing amount of active PAL, causing a further increase in the rate of synthesis of hydroxycinnamic acids. The increasing concentration of hydroxycinnamic acids progressively causes inhibition of PAL activity and induction of inactivation of PAL. This leads to a third phase in which both the PAL level and the rate of synthesis of hydroxycinnamic acids decline.

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