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CONTROL OF PHENYLALANINE AMMONIA-LYASE AND CINNAMIC ACID 4-HYDROXYLASE IN GHERKIN TISSUES

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; gherkin; cinnamic acid 4-hydroxylase; phenylalanine ammonia-lyase; induction.

Abstract—Blue light mediates a transient increase in the extractable activity of phenylalanine ammonia-lyase from both cotyledons and hypocotyls of etiolated gherkin seedlings, but concurrent changes in extractable cinnamic acid 4-hydroxylase activity only occur in cotyledons. Excision, followed by incubation in the dark, also results in stimulation of the lyase activity in both tissues, but the hydroxylase activity is only stimulated in cotyledons, again concurrently with the lyase. Stimulated levels of hydroxycinnamic acid esters are, however, only formed following light treatment, indicating the presence of another light-sensitive step in their biosynthesis. Treatment of gherkin tissues with 2-aminooxyacetic acid or α-aminooxy-β-phenylpropionic acid inhibits phenylalanine ammonia-lyase activity *in situ*, reduces the accumulation of hydroxycinnamic acid esters and presumably reduces the endogenous cinnamic acid pool. This treatment increases extractable lyase activity and delays its peak in activity. In cotyledons, these changes in the lyase are associated with concurrent and similar changes in extractable hydroxylase activity, whilst in hypocotyls the hydroxylase is relatively unaffacted. The increase in phenylalanine ammonia-lyase activity following excision of cotyledons and hypocotyls is prevented by cinnamic acid; in cotyledons the hydroxylase is similarly affected, but after a longer lag. Thus whilst cinnamic acid can modify the extractable activity of the lyase, it cannot itself mediate changes in the extractable activity of the hydroxylase.

INTRODUCTION

It is known [1] that light, particularly blue light, causes a rapid increase in the concentration of sugar esters of *p*-coumaric and ferulic acids in both the cotyledons and hypocotyls of etiolated gherkin seedlings. These hydroxycinnamic acid esters are the main phenolic products in these tissues, although flavonoids also accumulate in cotyledons [1] and their production presumably occurs via the phenylpropanoid pathway [2]. It is also known [3], at least in hypocotyls, that the accumulation of these products is associated with a transient increase in the extractable activity of phenylalanine ammoia-lyase (PAL) (EC 4.3.1.5), the first enzyme of the phenylpropanoid pathway.

The extractable activity of PAL in gherkin hypocotyls can also be increased by other treatments, for example, excision and incubation of segments in the dark [4]. There is, however, no increased accumulation of the hydroxycinnamic esters, unless light treatment is given [1]. This increase in PAL activity upon excision is prevented by cinnamic acid, the product of the PAL reaction and also by *p*-coumaric acid, the product of the second enzyme in the phenylpropanoid pathway, *trans*-cinnamic acid 4-hydroxylase (CA4Hase) (EC 1.14.13.11) [4, 5]. It is therefore thought that the increased PAL activity results from (hydroxy)cinnamic acid leaking out and being diluted by the incubation buffer, thus reducing the endogenous pool of (hydroxy)cinnamic acid [4, 5]. Further work of Engelsma [4] and Johnson *et al.* [5] using protein synthesis inhibitors and density labelling techniques respectively, suggests that the increase in PAL activity in excised hypocotyls is due to increased PAL synthesis, and that cinnamic acid acts as a feed-back repressor of PAL synthesis, in addition to inhibiting the activity of the enzyme [6].

The endogenous cinnamic acid pool can also be reduced, at least in theory, by treatment with specific inhibitors of PAL. Recently Amrhein and his colleagues [7, 8] have introduced compounds carrying the aminooxy group as potent inhibitors of phenylalanine deamination and transamination; these include α-aminooxyacetic acid (AOA) and L-α-aminooxy-β-phenylpropionic acid (AOPP). AOPP inhibits phenylalanine deamination far more specifically than transamination, whilst the reverse is the case for AOA. Unfortunately, the uptake of AOPP into plant tissue is much poorer than that of AOA, imposing a limitation on its use [8]. However, following treatment with AOPP, extractable PAL activity increases in both buckwheat and gherkin hypocotyls [9], presumably due to the absence of feed-back repression by cinnamic acid.

Changes in PAL activity are often associated with concurrent changes in the activities of other enzymes of the phenylpropanoid pathway, particularly CA4Hase and hydroxycinnamate:coenzyme A ligase [2]. Some

authors have assumed that such 'concurrent' changes in the two enzymes are evidence of 'co-ordinate' regulation, with PAL and CA4Hase being coded for by a common operon (e.g. [10]). In this paper, however, the term 'concurrent' is used purely descriptively, without any implications of function or mechanism. Indeed Engelsma [1, 11] has suggested that CA4Hase activity in gherkin hypocotyls is also stimulated by light, concurrently with PAL, but CA4Hase was measured using an indirect *in vivo* assay. This *in vivo* assay involved incubation of tissue segments with 10 mM *trans*-cinnamic acid and 1% glucose for 24 hr and used the *p*-coumaric acid glucose ester formed as a measure of CA4Hase activity. We have recently [12] isolated and characterized CA4Hase from gherkin tissues and shown it to be a microsomal P_{450} oxygenase, as are other CA4Hases [13], in contrast to PAL which is soluble (e.g. [14]). In this paper we have compared the effects of blue light and other treatments on the extractable activities of PAL and CA4Hase in gherkin hypocotyls, and also gherkin cotyledons, in a attempt to determine: (1) whether the effect of light on hydroxycinnamic acid ester production is due to an increased level of CA4Hase activity, and (2) the role of the cinnamic acid pool in controlling the activities of PAL and CA4Hase.

RESULTS

All enzyme activities presented in this paper refer to *extractable* activities, unless stated otherwise, and are expressed in terms of fresh weight of tissue, rather than protein content. This seems justified since, although the soluble protein content of gherkin cotyledons and hypocotyls gradually declines with age and during incubation following exision, there is no variation between the protein or water content of tissues subjected to different treatments (see below).

In each case the results presented are representative of at least three identical experiments. To check that the applied inhibitors actually inhibit PAL *in situ*, relative PAL activity in intact cells was estimated using the method developed by Amrhein and his colleagues ([7, 15]; see also Experimental).

Photocontrol of PAL and CA4Hase in intact tissues

PAL and CA4Hase are found in all parts of etiolated gherkin seedlings (Table 1). Exposure of 4-day-old seedlings to blue light for 3.5 hr causes a dramatic increase in PAL activity of cotyledons and the apical 1 cm segment of hypocotyls, but CA4Hase activity is stimulated significantly only in the cotyledons (Table 1). CA4Hase in hypocotyls remains at a constant level during 0–6 hr irradiation with blue light. In cotyledons, however, the blue-light-mediated changes in PAL and CA4Hase activities occur concurrently (Fig. 1) and the time course is similar to that of PAL in hypocotyls (Fig. 2 and ref. [3]). In hypocotyls the dark level of PAL relative to that of CA4Hase (see PAL/CA4Hase ratio) is noticeably lower than in cotyledons, but in roots there is a predominance of PAL (Table 1). It should be noted that it is particularly difficult to compare the absolute activities of these two enzymes since PAL is a soluble enzyme whilst CA4Hase is a microsomal enzyme and their recoveries may therefore differ. That the recovery of PAL in different tissues is similar is confirmed by an intact cell assay for PAL (see Methods). For example, in one set of plants the extractable activities of PAL in dark cotyledons and hypocotyls were 0.084 and 0.036 nmol product/min/g fr.wt, respectively (cotyledon/hypocotyl = 2.33). Using the intact cell assay, the ratio of activity cotyledons/hypocotyls was 2.11.

These results suggest that the light-mediated increase in hydroxycinnamic acid esters in cotyledons (see Introduction) may well be due to increased CA4Hase activity, but in hypocotyls this is clearly not the case since CA4Hase remains constant. The next question, therefore, was whether CA4Hase activity in cotyledons can be stimulated in the dark, i.e., when the phenolic end-products do not accumulate.

Effect of AOA on PAL and CA4Hase in intact tissues

The intact cell assay for PAL shows that spraying whole seedlings with 10 and 20 mM AOA inhibits PAL activity *in situ* by 95 and 100%, respectively, 2.5 hr after spraying; this is true for both hypocotyls and cotyledons. When tissues are extracted the AOA (or AOPP or cinnamic acid) is of course removed by desalting before assaying extractable PAL activity *in vitro*.

When seedlings left in the dark are sprayed with 10 mM AOA, the extractable activity of PAL in hypocotyls and cotyledons is not affected (e.g. Fig. 2, cotyledons). However, when sprayed with 20 mM

Table 1. The effect of 3.5 hr of blue light (L) on the extractable activities of PAL and CA4Hase in etiolated (D) gherkin tissues

Tissue	Treatment	PAL nmol/min g fr.wt	L/D	CA4Hase nmol/min g fr.wt	L/D	PAL/CA4Hase
Cotyledon	L	0.552	9.3	0.064	5.3	8.6
	D	0.059		0.012		4.9
Hypoctyl (Apical)	L	0.164	8.2	0.025	1.2	6.7
	D	0.020		0.020		1.0
Hypocotyl (Basal)	L	0.042	2.6	0.015	1.2	2.8
	D	0.016		0.013		1.3
Root	L	0.340	1.7	0.022	1.7	15.0
	D	0.200		0.03		15.7

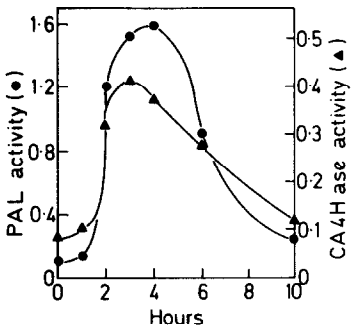

Fig. 1. Changes in PAL and CA4Hase activities in the cotyledons of etiolated gherkin seedlings during irradiation with blue light. Activities are expressed as nmol product/min/g fr.wt.

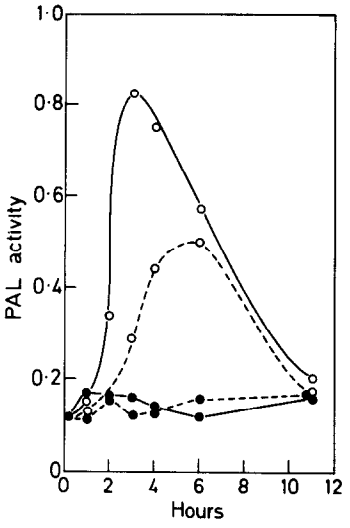

Fig. 2. Effect 10 mM AOA on extractable PAL activity in intact cotyledons either irradiated with blue light (○) or left in darkness (●). – – –, plus AOA; ——, minus AOA. Activity is expressed as nmol product/min/g fr.wt.

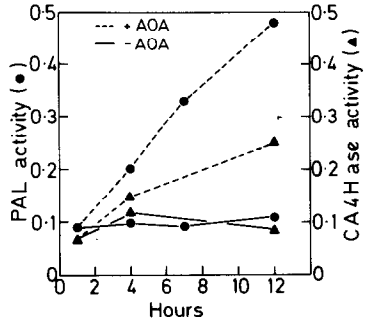

Fig. 3. Effect of 20 mM AOA on the extractable activities of PAL and CA4Hase in intact cotyledons in the dark. Enzyme activities are expressed as nmol product/min/g fr.wt.

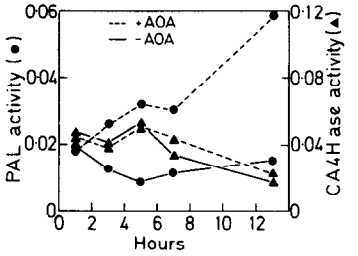

Fig. 4. Effect of 20 mM AOA on the extractable activities of PAL and CA4Hase in intact hypocotyls in the dark. Enzyme activities are expressed as nmol product/min/g fr.wt.

AOA there is a 3- to 5-fold increase in extractable PAL activity in both cotyledons (Fig. 3) and hypocotyls (Fig. 4). These results could be interpreted as follows: the cinnamic acid pool in the 10 mM AOA treatment is still large enough to repress PAL formation, whilst 20 mM AOA removes at least part of this repression, allowing an increased rate of PAL synthesis, this resulting in increased PAL activity. This is the simplest interpretation, following the arguments of Johnson *et al.* [5], and does not take account of the possible effects of cinnamic acid on PAL degradation or inactivation. Extractable CA4Hase activity is also unaffected by 10 mM AOA, but 20 mM AOA results in an increase in its activity in cotyledons (Fig. 3), but not hypocotyls (Fig. 4). Although 10 mM AOA does not affect extractable PAL activity in etiolated tissues, it does modify the time course of the blue-light-mediated increase, delaying the peak by *ca* 2 hr (Fig. 2 for cotyledons: see ref. [9] for hypocotyls). Thus the reduction in cinnamic acid concentration in the 10 mM AOA treatment is sufficient to change the time of decline in PAL activity.

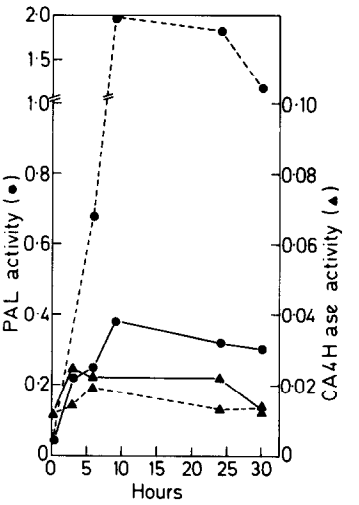

Fig. 5. Changes in extractable PAL and CA4Hase activities in excised 2 mm (– – –) and 1 cm (——) segments of hypocotyls incubated in the dark. The soluble protein content of hypocotyls declines from *ca* 2.58 mg/g fr.wt at excision to *ca* 1.90 mg/g fr.wt 30 hr after excision. The microsomal protein content remains at *ca* 0.70 mg/g fr.wt of hypocotyl. Enzyme activities are expressed as nmol product/min/g fr.wt.

Effect of excision on the extractable activity of PAL and CA4Hase

Incubation of 1 cm long hypocotyl segments in buffered solutions at 25° results in a rapid increase in PAL activity reaching a maximum at *ca* 9 hr, and then remaining constant for the next 20 hr (Fig. 5). With 2 mm hypocotyl segments the increase in PAL activity is much larger and the activity drops between 24 and 30 hr incubation (Fig. 5). The corresponding changes in CA4Hase activity are small and not significant (Fig. 5).

Incubation of excised cotyledons results in a large increase in PAL activity, again reaching a peak at *ca* 9 hr, but declining rapidly in the next 20 hr (Fig. 6); these changes are mimicked by somewhat smaller changes in CA4Hase activity (Fig. 7).

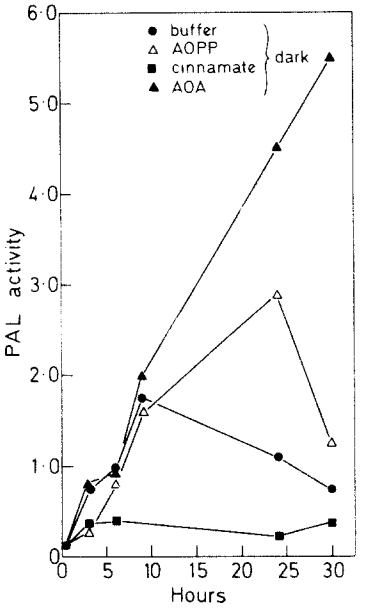

Fig. 6. Effect of 0.1 mM AOPP, 1 mM AOA or 1mM cinnamate on the extractable activity of PAL in excised cotyledons. The soluble protein content of cotyledons declines from *ca* 38.5 mg/g fr.wt at excision to *ca* 33.8 mg/g fr.wt 30 fr after excision. Enzyme activity is expressed as nmol cinnamate/min/g fr.wt.

Effect of cinnamic acid, AOPP and blue light on the activities of PAL and CA4Hase in excised tissues

In situ PAL activity in excised cotyledons and hypocotyls is completely inhibited following a 3 hr pre-incubation in incubation buffer containing added cinnamic acid (1 mM) (Table 2), as measured using the intact cell assay [15]. In the presence of 0.1 mM AOPP, on the other hand, significant inhibition (80–90%) of PAL activity *in situ* results only after a 5 hr pre-incubation with AOPP (Table 2), presumably due to its slower penetration [9]. The accumulation of hydroxycinnamic acid esters in excised tissue incubated in the dark for 24 hr is small but is reduced by 40% when 0.1 mM AOPP is included in the buffer (Table 3).

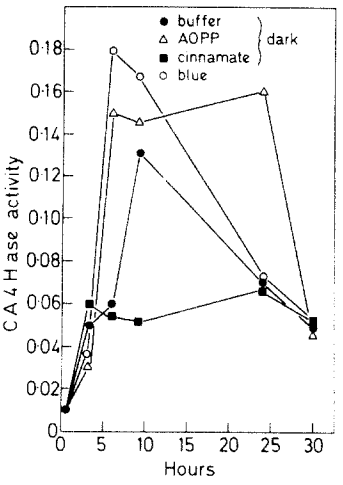

Fig. 7. Effect of various treatments on the extractable activity of CA4Hase in excised cotyledons. The microsomal protein content remains at *ca* 1.20 mg/g fr.wt. Other details as in Fig. 6.

Cinnamic acid significantly reduces extractable PAL activity within 3 hr in both excised hypocotyls and cotyledons, the effect being a little slower in the case of hypocotyls (Figs. 6 and 8). In hypocotyls, AOPP causes a linear increase in PAL activity for 30 hr thus exceeding the activity in buffer alone, which reaches a plateau at 9 hr (Fig. 8). The situation in cotyledons is similar in the presence of AOPP but PAL activity reaches a peak at 24 hr after excision and then declines (Fig. 6). AOA (1 mM) causes an even larger increase in PAL levels in cotyledons (Fig. 6), which continues for at least 30 hr.

In hypocotyls, CA4Hase activity is unaffacted by these treatments and remains in the range 0.014 to 0.032 nmol product/min/g fr.wt (Fig. 9). In cotyledons, on the other hand, cinnamic acid inhibits the rise in

Table 2. Inhibition of PAL by cinnamic acid and AOPP in intact cells

Hr pre-incubation	Compound tested	% Inhibition Hypocotyl	% Inhibition Cotyledon
3	AOPP	45	35
5	AOPP	78	96
15	AOPP	88	91
3, 5 or 15	Cinnamic acid	100	100

PAL activity in excised hypocotyls or cotyledons is assayed using the intact cell assay in the presence of either 0.1 mM AOPP or 1mM cinnamic acid following pre-incubation in the same substance in the dark. The relative activities in cotyledons and hypocotyls are 3.3–4.8 : 1.

Table 3. Effect of 0.1 mM AOPP on the accumulation of hydroxycinnamic acids in excised cotyledons incubated in darkness

Hr incubation	Absorbance (315 nm)/cotyledon pair Buffer	AOPP
0	0.30	—
8.5	0.33	0.25
24.0	0.62	0.35

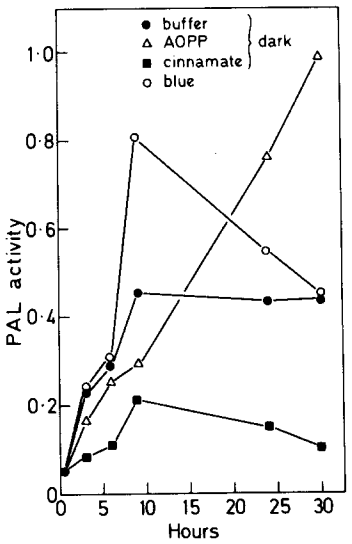

Fig. 8. Effect of various treatments on the extractable activity of PAL in excised 1 cm hypocotyl segments. Other details as in Fig. 6.

CA4Hase activity, after a lag of 6 hr (Fig. 7), but by 24 hr the activity is similar to the buffer control (cf. Fig. 6 where PAL is inhibited 84% at 24 hr). There is no evidence for substrate induction of CA4Hase (Fig. 7). The CA4Hase time course in AOPP-treated cotyledons is similar to that of PAL in that the peak is delayed to *ca* 24 hr, but the stimulation over control is smaller. CA4Hase in cotyledons was also stimulated by AOA but this was more variable, sometimes increasing to a peak at 9 hr and then declining, and sometimes increasing steadily with time, like PAL.

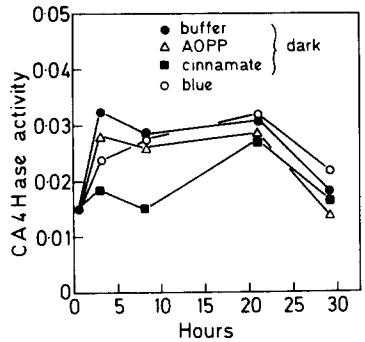

Fig. 9. Effect of various treatments on the extractable activity of CA4Hase in excised 1 cm hypocotyl segments. Other details as in Fig. 6.

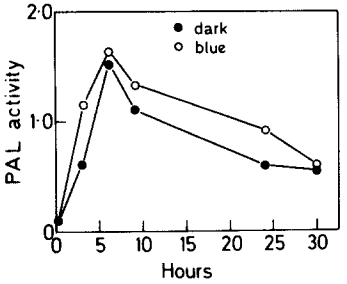

Fig. 10. Effect of blue light on the extractable activity of PAL in excised cotyledons; activity expressed as nmol cinnamate/min/g fr. wt.

The effect of blue light on PAL activity in excised tissue is much smaller than in intact tissue (Fig. 9, hypocotyls; Fig. 10, cotyledons: note that peak activity occurs at 6 hr in this figure due to a slightly increased temperature during incubation [16]. Blue light does not stimulate CA4Hase activity in excised hypocotyls, as was found in intact hypocotyls, but CA4Hase activity extracted from excised cotyledons was stimulated, again less than in intact cotyledons.

DISCUSSION

Our results show that blue-light treatment of etiolated gherkin seedlings mediates a transient and concurrent increase in the extractable activities of both PAL and CA4Hase in cotyledons, but only PAL is increased in hypocotyls. However, in cotyledons concurrent increases in PAL and CA4Hase also occur in the dark, without increased accumulation of the products, sugar esters of hydroxycinnamic acids. Thus, it appears that the production of these products is restricted in the dark by a subsequent rate-limiting step, which is stimulated by light; it is suggested that when Engelsma [11] was attempting to measure CA4Hase activity using an *in vivo* assay, an assay which measured the end-product of a number of reactions, he instead was measuring the effects of light on this step. Another enzyme whose activity is often stimulated by light, usually concurrently with PAL, is *p*-coumaroyl coenzyme A ligase [2]; this may be the enzyme involved in gherkin tissues.

Despite large changes in PAL activity in gherkin hypocotyls, CA4Hase activity in this tissue was relatively unaffected. This is somewhat unexpected since the activities of these two enzymes are usually modified concurrently [2]. However, the fact that in etiolated gherkin seedlings CA4Hase activity relative to PAL activity is greater in hypocotyls than in cotyledons may suggest that CA4Hase activity in hypocotyls is sufficient to cope with the increased flux through the pathway following treatment with light. Indeed, in light-treated seedlings the ratio of the activities of the two enzymes is similar in both tissues. In the light-treated tissues, however, PAL activity is much greater than CA4Hase activity (see Table 1), a situation which is not uncommon [17].

The results with excised tissue confirm the previous finding [4, 5] that extractable PAL activity in hypocotyls increases following excision, and extend the observation to cotyledons. Exogenous cinnamate

reduces the excision response of PAL in both cotyledons and hypocotyls, whilst treatment with AOA or AOPP, which specifically inhibit PAL activity *in situ*, and thus should reduce the cinnamic acid pool, results in a prolonged increase in extractable PAL activity, and a later peak of activity. These results support the suggestion [4, 5] that cinnamic acid, or its products, is a feed-back repressor of PAL.

In cotyledons the activities of PAL and CA4Hase changed similarly, if not concurrently, in response to various treatments. Thus treatments which are thought to reduce the endogenous cinnamic acid pool (AOA, AOPP, excision) increase both PAL and CA4Hase activities, whilst addition of cinnamic acid to excised cotyledons reduces the activities of both enzymes. There is no evidence, therefore, for the induction of CA4Hase by its substrate, cinnamic acid, and cinnamic acid does not appear to be responsible for the concurrent changes in PAL and CA4Hase activities in cotyledons.

The effect of cinnamic acid on PAL in cotyledons is faster than its effect on CA4Hase, suggesting that *p*-coumaric acid, and not cinnamic acid, is responsible for the reduction in CA4Hase activity, in agreement with the situation in potato tissue [18]. It is known that *p*-coumaric acid does not inhibit either gherkin PAL or CA4Hase *in vitro* [6, 12] and thus does not have a role in the fine control of these enzymes. However, *p*-coumaric acid is thought to repress PAL synthesis [4], as well as reducing the total extractable activity (synthesis?) of CA4Hase [18]. It would be useful to measure both the concentrations of the individual phenolics and the activities of PAL and CA4Hase in gherkin tissues subjected to various treatments, and then to compare the effects of exogenous additions of these individual phenolics on extractable activities of PAL and CA4Hase in an attempt to determine which phenolic(s) is important in controlling these two enzymes.

It has been suggested (see [2]) that the accumulation of (hydroxy)cinnamic acid induces the synthesis or release of a proteinaceous PAL inhibitor, in addition to repressing PAL synthesis. A reversible proteinaceous inhibitor of PAL has indeed been isolated from gherkin cotyledons and hypocotyls [14], but this inhibitor also inhibits CA4Hase. Thus, since changes in the activities of PAL and CA4Hase in response to various treatments are different in the two tissues, the role of the proteinaceous inhibitor in these changes is not clear. What is clear, however, is that the popular theory (e.g. [10]) in which PAL and CA4Hase are thought to be controlled on a single operon, thus allowing co-ordinate regulation of both enzymes, is also too simple, and that presently there is no good explanation for the observed concurrent control of these enzymes in one tissue and not in another. In addition, it appears that in gherkin cotyledons the concurrent increase in PAL and CA4Hase activities in response to light, and other stimuli, is due to separate effects on the two enzymes.

EXPERIMENTAL

Plant material and treatment conditions. Gherkin seedlings (*Cucumis sativus* cv Venlo Pickling, Suttons, Reading, U.K.) were grown in 22 × 15 cm plastic boxes in darkness at 25 ± 1° for 4 days. All manipulations of seedlings were undertaken under a green safety light. Intact plants were sprayed with H_2O or AOA (20 cm^3/box) and returned to darkness. Excised tissue was incubated in 10 cm Petri dishes in 8 cm^3 5 mM K-Pi (pH 5.5) and 100 $\mu g/cm^3$ penicillin [4] on Whatman No. 1 filter paper: 30 × 1 cm long hypocotyl segments, including plumular hook, or 30 cotyledon pairs per dish. Cinnamic acid and AOPP were added at a final concn of 1 and 0.1 mM, respectively. Intact plants or tissue segments were irradiated with narrow band blue light (1.1 W/m^2) [19].

Extraction and assay of enzymes. Enzymes were extracted from either 60 hypocotyl segments or 60 cotyledon pairs (*ca* 2 g fr. wt). For the extraction of phenylalanine ammonia-lyase, tissue was extracted in 3 cm^3 0.1 M K-Pi (pH 7.5) containing 10^{-3} M glutathione, using a pestle and mortar. After filtration through 1 layer of wet Miracloth, the homogenate was centrifuged at 20 000 g for 15 min. The supernatant (1.5 cm^3) was desalted on a Sephadex G-25 column pre-equilibrated with the extraction buffer and assayed immediately by incubation with 1.0 cm^3 0.1 M borate (pH 8.8) containing 0.1 mM L-[4-^{3}H]-phenylalanine (1 Ci/mol) (Radiochemical Centre, Amersham) and 10^{-4} glutathione [11]. The assay was at 25° for 60 min. Cinnamic acid 4-hydroxylase (CA4Hase) was extracted and assayed as described previously [12, 14]. The microsomal fraction, which contained the CA4Hase, was resuspended in resuspension buffer and either assayed immediately or stored in liquid N_2; freezing caused no loss of enzyme activity. CA4Hase was not inhibited by AOPP or AOA *in vitro*.

Intact cell assay for phenylalanine ammonia-lyase (PAL). This was undertaken basically as described by Amrhein *et al.* [7, 15] to test the efficiency of inhibition by cinnamic acid, AOA and AOPP *in situ* under the chosen experimental conditions. The substrate used, however, was L-[2,3-^{3}H(N)]-phenylalanine (Radiochemical Centre, Amersham) and not L-[-^{3}H(N)]-phenylalanine. Eight hypocotyl segments or 8 cotyledon pairs (*ca* 0.25 g) were incubated in 0.5 cm^3 0.15 M K-Pi (pH 5.5) containing 2.4×10^{-4} M L-[2,3-^{3}H(N)]-phenylalanine (19.2 Ci/nmol) in 25 cm^3 Erlenmeyer flasks with constant shaking at 25°. Following sublimation of ^{3}HOH from 50 mm^3 of the medium, 10 cm^3 of scintillation fluid (7.5 g 2,5-diphenyloxazole, 1 dm^3 toluene, 500 cm^3 Triton X-100) were added for the determination of ^{3}H activity. In this assay the N^3HH_2 evolved exchanges with tissue water, and subsequently with buffer water, forming ^{3}HOH; this ^{3}HOH is sublimed and counted and represents PAL activity in the intact cells. Uptake of substrate into the tissue and the stereospecificity of the reaction was checked basically as described in ref. [15].

Other assays. Protein was determined by the method of Lowry with BSA as standard. Hydroxycinnamic acid esters of *p*-coumaric and ferulic acids were assayed according to ref. [1].

Chemicals. α-Aminooxyacetic acid, semihydrochloride, was obtained from Sigma, and L-α-aminooxy-β-phenylpropionic acid was a kind gift from Dr. J. S. Morley, ICI Pharmaceutical Division, Macclesfield, Cheshire, U.K.

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