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KIEVITONE PRODUCTION AND PHENYLALANINE AMMONIA-LYASE ACTIVITY IN COWPEA

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Key Word Index—*Vigna sinensis*; Leguminosae; cowpea; isoflavanoids; phytoalexin biosynthesis; kievitone; phenylalanine ammonia-lyase.

Abstract—Kievitone, a phytoalexin of cowpea, increases following treatment of hypocotyls with $CuCl_2$, actinomycin D, cycloheximide or UV-irradiation. Cellular browning accompanies this increase. Kievitone production is restricted to the regions containing necrotic cells, whereas phenylalanine ammonia-lyase (PAL) activity increases throughout the hypocotyl. Kievitone biosynthesis is unlikely to be regulated by control of PAL activity.

INTRODUCTION

Following fungal and viral inoculation, cowpea (*Vigna sinensis* Endl.) produces several phytoalexins, including the isoflavanoids kievitone, phaseollin and phaseollidin [1, 2]. In other legumes phytoalexin biosynthesis has been induced by abiotic treatments [3–9], and it has been proposed that phytoalexin production depends on *de novo* formation of messenger RNA, following activation of specific host genes, including that for phenylalanine ammonia-lyase (PAL) [10, 11]. However, other workers have failed to find an increase in *m*RNA synthesis following treatment with compounds which induce phytoalexin synthesis in pea [12, 13] and bean [14]. Like other isoflavanoid phytoalexins [15–17], kievitone appears to be synthesized via the shikimic acid pathway, since L-phenylalanine [U-^{14}C] is incorporated into kievitone in cowpea leaves following fungal inoculation (Munn, unpublished results). The relationship in cowpea between changes in PAL activity and production of the phytoalexin kievitone is described here.

RESULTS

Following topical application of $CuCl_2$, actinomycin D, or cycloheximide solutions of various concentrations to their upper surface, excised etiolated hypocotyls were incubated before being split longitudinally to separate the upper half (part bearing lesions) from the lower half. Measurements of PAL activity and kievitone concentration in each part of the hypocotyl were made after 24- and 48 hr incubation respectively since while changes in PAL activity were readily detectable within 24 hr, changes in kievitone concentration could not be detected consistently until after 48 hr incubation.

Following application of $CuCl_2$ (Table 1), kievitone was detected in both upper and lower halves, reaching a maximum concentration in the upper half with 1·7 mg/ml $CuCl_2$. With 5·1 mg/ml, the kievitone content declined in the upper, but increased in the lower half of the hypocotyl. PAL activity in both halves of the hypocotyl increased with $CuCl_2$ concentration up to 1·7 mg/ml. However 5·1 mg/ml $CuCl_2$ induced a marked increase in the upper half, but not in the lower half (Table 1).

After actinomycin D treatment (Table 1), kievitone was detected only in the upper half of the hypocotyl and reached a maximum concentration with 10 μg/ml actinomycin D. Higher concentrations of the antibiotic reduced kievitone synthesis. PAL activity increased following treatment with concentrations of actinomycin D up to

Table 1. PAL activity and kievitone concentration in cowpea hypocotyls treated with $CuCl_2$, actinomycin D, or cycloheximide.

Compound	Concentration (μg/ml)	PAL activity* Upper‡	PAL activity* Lower	Kievitone Concentration† (μg/g dry wt) Upper	Kievitone Concentration† (μg/g dry wt) Lower
Water (control)		6·3 ± 0·2	6·3 ± 0·2	0	0
$CuCl_2$	1·7	8·4 ± 0·3	7·8 ± 0·3	16·0	0
	17	12·0 ± 0·2	9·3 ± 0·6	74·0	0
	1700	13·8 ± 0·3	12·5 ± 0·5	183·0	20·0
	5100	24·6 ± 0·8	11·7 ± 0·3	100·0	74·0
Actinomycin D	0·1	11·6 ± 0·2	11·0 ± 0·2	15·5	0
	1·0	11·7 ± 0·7	11·2 ± 0·2	24·0	0
	10	12·7 ± 0·4	11·4 ± 0·3	81·0	0
	20	11·3 ± 0·1	9·6 ± 0·5	15·5	0
	50	7·8 ± 0	5·4 ± 0·2	15·5	0
Cycloheximide	0·1	4·8 ± 0·1	4·3 ± 0·1	0	0
	1	6·0 ± 0·6	4·9 ± 0·2	0	0
	10	5·2 ± 0	4·1 ± 0·3	20·0	0
	100	8·5 ± 0·2	5·3 ± 0·1	59·0	0
	500	9·1 ± 0·5	7·2 ± 0·8	0	0

* Conversion of L-phenylalanine [ring-4-^{3}H] to cinnamic acid [ring-4-^{3}H], dpm/hr/mg protein × 10^3, assayed 24 hr after treatment (mean of four determinations ± s.e.).

† Assayed 48 hr after treatment (mean of two determinations).

‡ Hypocotyls were split longitudinally to separate the upper and lower halves.

10 μg/ml, and activity in the upper and lower halves was similar (Fig. 1, Table 1).

At low concentrations of cycloheximide, 0·1–10 μg/ml, PAL activity was lower than in the controls, and only slight increases were observed with 0·1 and 0·5 mg/ml. In contrast, kievitone, which as with most other treatments was only detected in the upper half, was present following treatment with both 10 and 100 μg/ml cycloheximide. With 10 μg/ml cycloheximide, a level which causes kievitone production, the PAL activity in the hypocotyls had not increased. The level of activity after 24

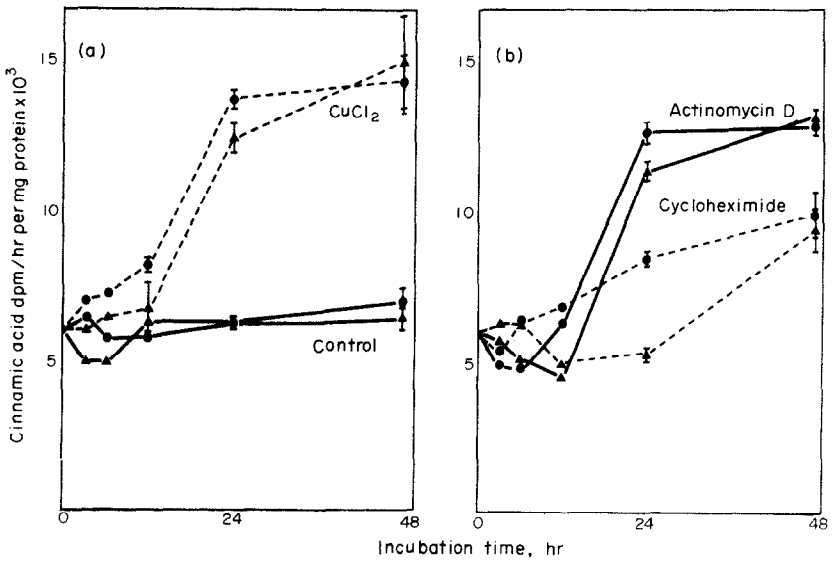

Fig. 1. PAL activity in cowpea hypocotyls treated with actinomycin D (10 μg/ml), $CuCl_2$ (1·7 mg/ml) or cycloheximide (0·1 mg/ml). PAL activity (conversion of L-phenylalanine [ring-4-^{3}H] to cinnamic acid [ring-4-^{3}H] was assayed 0–48 hr after treatment (mean of four determinations ± s.e.). Hypocotyls were split longitudinally to separate the upper (●) and lower (▲) halves.

Table 2. Kievitone concentrations in the upper and lower halves of etiolated hypocotyls at various times after topical application of chemicals (data are the mean of two results)

Treatment	Part of hypocotyl	Kievitone concentration (μg/g dry wt.) 0 hr	12 hr	24 hr	48 hr	72 hr
Water	Upper	0	0	0	0	0
	Lower	0	0	0	0	0
$CuCl_2$ (1·7 mg/ml)	Upper	0	10	70	180	170
	Lower	0	0	0	20	50
Actinomycin D	Upper	0	0	22	83	60
(10 μg/ml)	Lower	0	0	0	0	0
Cycloheximide	Upper	0	0	20	60	50
(100 μg/ml)	Lower	0	0	0	0	0

and 48 hr was similar (5200 ± 0 and 4500 ± 200 dpm/hr/mg protein, respectively) and at both times these PAL levels are lower than those of the corresponding H_2O-treated controls (6300 ± 200 and 6400 ± 200 dpm/hr/mg protein).

All treatments produced cellular browning under the drops, and peaks in kievitone concentration corresponded with the most intense browning. High concentrations of $CuCl_2$ induced browning throughout the hypocotyl.

Kievitone concentration and PAL activity were also measured at various times following treatment with $CuCl_2$, actinomycin D, or cycloheximide at the concentration inducing the highest production of kievitone (Table 2). Following $CuCl_2$ treatment (1·7 mg/ml), kievitone was detected in the upper half of the hypocotyl at 12 hr, increasing to a maximum concentration at 48 hr, whereas in the lower half none was detected until after 48 hr. Following treatment with actinomycin D (10 μg/ml) or cycloheximide (100 μg/ml), maximum production of kievitone also occurred after 48 hr but was restricted to the upper half. H_2O-treated controls accumulated no kievitone. There was no significant change in PAL activity in control hypocotyls from 0–48 hr, whereas $CuCl_2$-treated tissue had more than twice the activity of control tissue at 48 hr, the greatest increase occurring between 12 and 24 hr (Fig. 1a). Actinomycin D (Fig. 1b) gave similar results to $CuCl_2$ treatment, but cycloheximide (Fig. 1b) induced only a small steady increase in PAL activity up to 48 hr. In all cases activities in the lower half were less after the shorter incubation times, but by 48 hr there was no difference in the PAL activity of the two parts of the hypocotyl.

Kievitone concentration and PAL activity increased in whole etiolated hypocotyls irradiated with UV light at 254 nm after irradiation times as short as 2·5 min (Fig. 2). Maximum kievitone accumulation occurred following 30 min irradiation, whereas maximum PAL activity was found with 40 min irradiation. Increases in kievitone were paralleled by increases in cellular browning, which varied from small flecks at 2·5 min to extensive brown patches at 30 min. After longer periods of irradiation, hypocotyls showed deformation and loss of turgidity, but no surface browning.

DISCUSSION

The results show that kievitone can be induced in cowpea by abiotic treatments and there is a close association between its accumulation and cellular browning. Phytoalexins can also be induced in cowpea by mechanical wounding [1] and this is in agreement with the hypothesis that phytoalexins accumulate in response to general metabolic changes accompanying or following cell

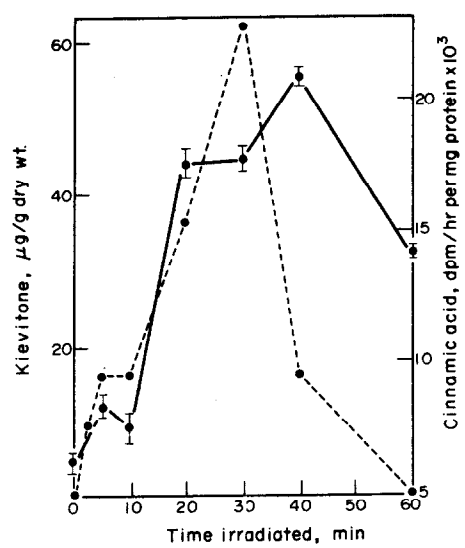

Fig. 2. PAL activity and kievitone concentration in cowpea hypocotyls following UV-irradiation. PAL activity (conversion of L-phenylalanine [ring-4-^{3}H] to cinnamic acid [ring-4-^{3}H]) was assayed 24 hr after irradiation for 0–60 min at 254 nm (mean of four determinations ± s.e.) (——). Kievitone (– – – –) was measured 48 hr after irradiation (mean of two determinations).

damage or death [18, 19]. If PAL is involved in regulating kievitone biosynthesis then changes in PAL activity would be expected to precede changes in kievitone concentration. Chemical treatments induced increases in PAL activity after 12 hr whereas only in $CuCl_2$-treated hypocotyls was kievitone detected at this time. Measurements of PAL activity were not made beyond 48 hr in these studies and it is unclear whether the activity drops after this time. There is also a spatial separation of PAL activity and kievitone synthesis since, except with $CuCl_2$, kievitone is restricted to the upper half of the hypocotyl (containing the necrotic cells) whereas increased PAL activity is not. However with higher concentrations of $CuCl_2$ or longer incubation times, both browning and kievitone were detected in both parts indicating transport of Cu^{2+} through the tissue in amounts sufficient to induce browning and kievitone synthesis as well as increased PAL activity. Rathmell [20] also observed a spatial and temporal separation of phaseollin biosynthesis and PAL activity in infected bean, suggesting that phytoalexin synthesis may be restricted to the necrotic cells whereas increased PAL activity (which may depend on new protein synthesis [7]) can occur outside the necrotic area.

Cycloheximide causes increased PAL activity (derived from a pool of inactive enzyme) in etiolated seedlings of gherkin [21] but inhibits the accumulation of the enzyme in light-treated potato tuber disks [22] and other systems [23, 24]. This compound has also been reported to prevent browning [22]. In cowpea, only a slight increase was observed following cycloheximide treatment (100 μg/ml) and kievitone synthesis was enhanced even at a concentration of cycloheximide (10 μg/ml) which reduced PAL activity below that of the control. It is therefore possible that basal levels of PAL are sufficient to allow kievitone synthesis, although further studies of cycloheximide treatment in conjunction with other treatments are necessary to confirm this. The control of PAL activity is clearly a complex mechanism [11, 25], and changes in its activity may not reflect other events occurring during phytoalexin induction. Since PAL is involved in numerous biosynthetic pathways, it is unlikely that regulation of its activity is important in the control of phytoalexin production [26]. Control of isoflavanoid biosynthesis may depend on the regulation of activity of enzymes occurring later in the pathway.

Phytoalexin synthesis is induced by UV-irradiation not only in cowpea, but also in other legumes, where it may [9] or may not [6] be accompanied by browning. It has been proposed that induction occurs as a consequence of gene activation through pyrimidine dimer formation [6] although there is no evidence that this would lead to direct activation of the specific genes. Our results show that kievitone induction in cowpea is always accompanied by cellular browning and necrosis, suggesting that this phytoalexin is induced by UV-irradiation and other treatments as a result of processes accompanying cellular damage or death. Whether or not protein and RNA synthesis are necessary for induction remains unclear.

EXPERIMENTAL

Cultivation and treatment of plants. Etiolated hypocotyls were excised from seed grown in the dark for 6 days at 25°, 50% RH, in a growth chamber and placed horizontally on glass rods in shallow plastic boxes lined with wet paper tissues. Solns of $CuCl_2$, actinomycin D or cycloheximide were spotted on the upper surface of each hypocotyl (10 × 3 μl drops per hypocotyl). After incubation (25°, 100% RH, in dark) hypocotyls were split along their long axis to separate the upper half (part bearing lesions) from the lower half prior to assay. For expts on the effect of UV, unexcised seedlings were irradiated on both sides (10 cm from Camag Universal Lamp, 254 nm), incubated as above and whole excised hypocotyls homogenised prior to assay.

Kievitone assays. Tissue was weighed, homogenised in 50 ml 95% EtOH and filtered through muslin. The residue was rehomogenised 2×, the combined filtrates centrifuged (3000 *g*, 15 min) and the supernatant reduced *in vacuo* (40°) to *ca* 10 ml. H_2O (10 ml) was added and the suspension extracted with 3 × 50 ml Et_2O after acidification to pH 2·5, followed by TLC of the conc Et_2O phase in $CHCl_3$–EtOH (20:1), C_6H_6–1,4 dioxan–HOAc (8:1:1) and toluene–HCOOEt–HCOOH (7:2:1) using authentic kievitone as marker. Kievitone was determined by A at 293 nm (log ϵ 4·22) [19].

PAL assays. Me_2CO powders [27] were prepared by homogenizing for 1 min (10 ml/g fr. wt, −20°). The homogenates were filtered (5 μm Teflon), rinsed with an equal vol of cold Me_2CO and dried on the filter in an airstream for 15 min. PAL was extracted from the powder with ice-cold 0·01 M borate buffer pH 8·8 containing 0·01 M mercaptoethanol (50 mg powder/10 ml). The suspension was kept at 0° for 1 hr with occasional stirring and the extract filtered through glass-fibre paper. For assay [28] 1 ml of the supernatant was added to 1 ml of 2 mM L-phenylalanine containing 0·25 μCi of L-phenylalanine [ring 4-^{3}H] (Radiochemical Centre, Amersham, Bucks.). After incubation (35°, 1 hr) 1 ml 20% TCA was added, the mixture centrifuged and the supernatant decanted. Cinnamic acid was extracted into 3 ml toluene and the toluene phase washed with 3 ml 0·5 M HCl to remove dissolved phenylalanine. Two ml of the upper phase was added to 8 ml scintillation fluid and counted. Protein was determined by the method of Lowry *et al.* [29].

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