INDUCTION OF PHENYLPROPANOID PATHWAY ENZYMES IN ELICITOR-TREATED CULTURES OF *CEPHALOCEREUS SENILIS*

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Abstract—Treatment of old-man-cactus (*Cephalocereus senilis*) suspension cultures with chitin elicits synthesis of an aurone phytoalexin, cephalocerone. Elicitor-induced *de novo* synthesis of cephalocerone was demonstrated by incubating elicited cactus cultures with [³H]phenylalanine; this resulted in the labelling of five induced phenolic compounds including cephalocerone. Increased extractable activities of the phenylpropanoid pathway enzymes phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and chalcone isomerase (CHI) accompanied the synthesis of cephalocerone. CHS and PAL, which are both involved in the biosynthesis of cephalocerone, showed maximum activity at 12 and 24 hr post-elicitation, respectively. CHS and CHI activities catalysing the synthesis and subsequent isomerization of 2′,4′,6′-trihydroxychalcone were present in the cell cultures, consistent with the formation of cephalocerone via a chalcone with no B-ring substituents.

INTRODUCTION

Many temperate crop plants such as potato, rice and cotton have long been known to accumulate phytoalexins in response to infection or elicitation [1]. Several reports have suggested that the accumulation of such antimicrobial compounds may be an important factor in resistance to plant pathogens by these crop plants [2, 3]. In many legumes, the most potent phytoalexins are products of the flavonoid biosynthetic pathway including such isoflavonoids as medicarpin, phaseollin and glyceollin; much is known regarding their synthesis and the enzyme induction phenomena on which it depends [4].

In contrast, there is little known about the chemical responses of desert adapted plants to microbial infections. Constitutive cactus compounds that deter feeding of cactophilic yeast and associated *Drosophila*, both of which metabolize necrotic cactus tissue, have been the focus of considerable study of chemical–microbe and chemical–insect interactions [5, 6]. Recent studies with *Cephalocereus senilis* (old-man-cactus), a species from southern Mexico, established that an aurone accumulated after elicitation of the cactus cell suspension with chitin and that the purified aurone inhibited growth of a number of gram negative bacteria [7]. It has since been observed that this aurone, cephalocerone (1), inhibits the growth of *Erwinia cacticida* [8], a cactus pathogen of southwest U.S. and Mexico which appears to be responsible for the common soft rot infection [9]. In order to define the mechanisms underlying induction of a phytoalexin in a member of the Cactaceae, we now present evidence for the *de novo* synthesis of cephalocerone in elicitor treated *C. senilis* cell suspensions. This is accompanied by increases in the extractable activities of key enzymes in the flavonoid pathway leading to aurone biosynthesis (Fig. 1). We also examined the substrate specificity of chalcone synthase (CHS) and chalcone isomerase (CHI) in relation to the B-ring substitution pattern of cephalocerone.
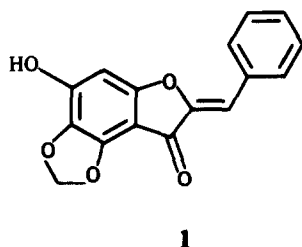
RESULTS AND DISCUSSION

Aurone labelling

Cell suspension cultures of *C. senilis* were treated with elicitor and [³H]phenylalanine, and cell extracts prepared as described in the Experimental. [³H]Phenylalanine was the only radiolabelled peak in the 0.01 M HCl rinses. The incorporation of tritium label into metabolites at different times post-elicitation is shown in Fig. 2. Radioactive peaks from the phenolic fraction are

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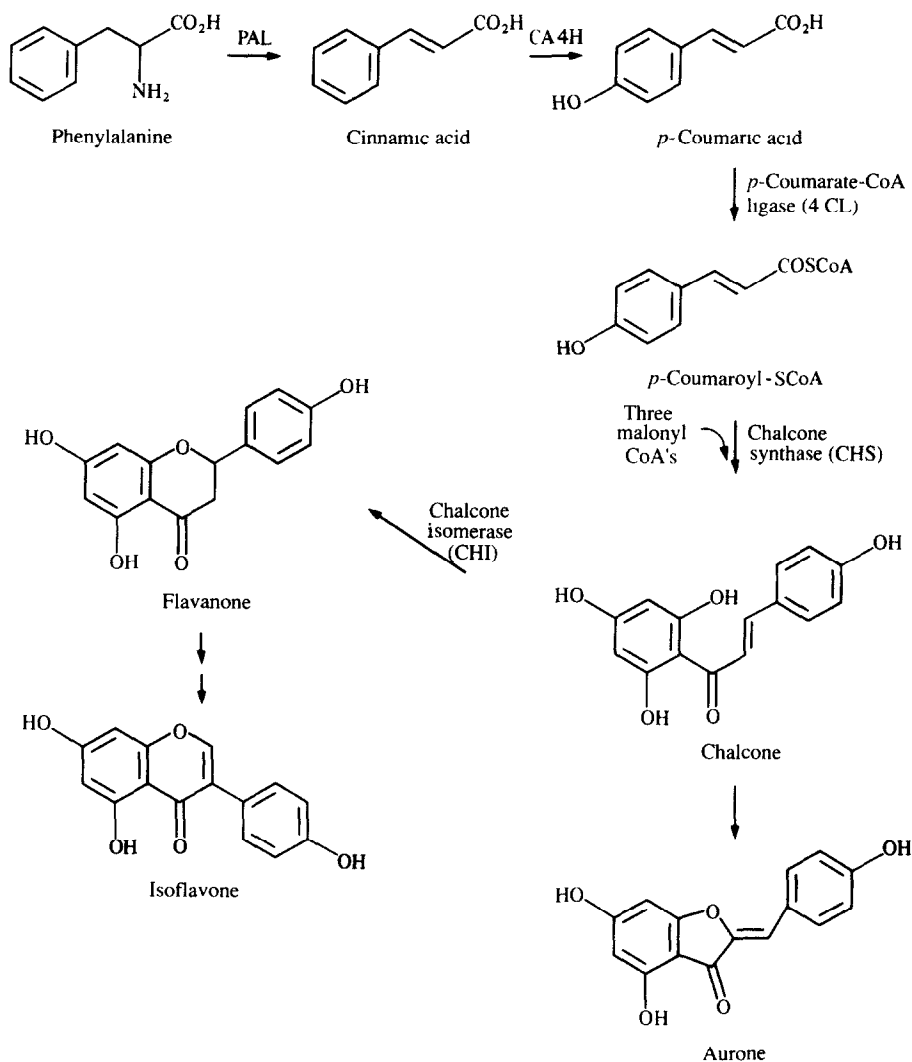‡Author to whom correspondence should be addressed.

Fig. 1. Flavonoid biosynthetic pathway leading to two classes of phytoalexins, isoflavones and aurones.

reported as the per cent of total administered [³H]phenylalanine. Peaks 2, 6, 10 and 12 show an increase in incorporation following elicitation of the suspension culture (6–24 hr, Table 1). Cephalocerone (peak 6) was identified by radioassay on the basis of co-chromatography with an authentic standard. The radio-tracer HPLC studies showed that there was no accumulation of several intermediates or possible alternative products in the pathway, namely, cinnamic acid, p-coumaric acid, 2',4,4',6'-tetrahydroxychalcone, 4',5,7-trihydroxyflavanone and 4,4',6-trihydroxyaurone.

Elicitor induction of enzymes of flavonoid biosynthesis

The activities of PAL, CHS and CHI (Fig. 1) were measured in old-man-cactus liquid suspension culture following the addition of chitin. Control flasks were treated with 5 ml of sterile H_2O. The activity measurements reported are the average of two replicates; the standard deviation for each time point is shown in Figs 3–5. Twenty-four hours after the cells were elicited, the maximum level of spectrophotometrically deter-

mined PAL activity was 40 times greater than recorded for the control cells. Use of an alternative PAL assay, based on incorporation of [¹⁴C]phenylalanine into cinnamic acid, confirmed the correlation between changes in absorbance at 290 nm and cinnamic acid formation (data not shown) and yielded the same induction profile.

p-Coumaroyl CoA is normally used as a substrate for CHS in flavonoid enzyme induction studies since most flavonoids have a 4'-hydroxyl group; however, as cephalocerone has an unsubstituted B-ring, cinnamoyl CoA was also tested as a possible substrate. In the presence of malonyl CoA, the two potential substrates were converted to their corresponding chalcones most rapidly in extracts from cells 12 hr after elicitation. With p-coumaroyl CoA as the substrate a 20-fold increase in activity was recorded in elicited cells, and cinnamoyl CoA resulted in a six-fold activity increase; the activity decreased with both substrates between 12 and 24 hr post-elicitation. As both cinnamoyl CoA and p-coumaroyl CoA were converted to chalcones, there is either dual specificity by CHS for these substrates or there are two distinct enzymes responsible for the formation of the two different chal-
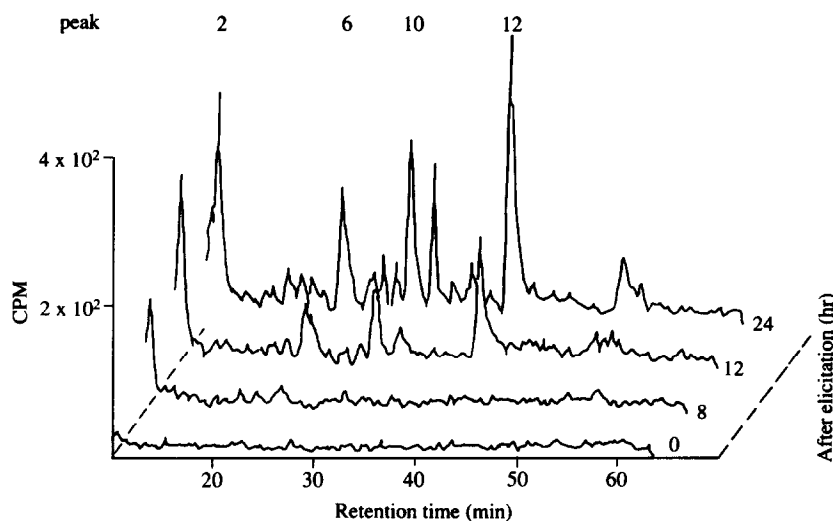
Fig. 2. HPLC profile of radioactive compounds extracted from elicited old-man-cactus cultures 0, 8, 12 and 24 hr after feeding 50 μl L-[2,6-^{3}H]phenylalanine (2.11 TBq mmol^{-1}, 0.88 nM); 50 μl of the phenolic extract were injected into the HPLC.

Table 1. Metabolites of [2,6-^{3}H]phenylalanine in elicited old-man-cactus cultures

Time (hr)	% Administered radioactivity				
	Peak 1	Peak 2	Peak 6	Peak 10	Peak 12
4	23.5 ±0.5	n.d.	n.d.	n.d.	n.d.
6	30.0 ±0.6	0.48 ±0.05	0.12 ±0.06	0.06 ±0.06	n.d.
8	38.4 ±1.2	0.48 ±0.04	0.24 ±0.024	0.18 ±0.06	0.3 ±0.06
12	37.2 ±1.8	0.84 ±0.24	0.48 ±0.06	0.48 ±0.28	1.14 ±0.18
24	22.2 ±1.8	1.26 ±0.30	0.60 ±0.12	0.96 ±0.36	1.74 ±0.06

Results represent the mean of duplicate analysis with standard deviation.
n.d.: not detected.

cones. The induction kinetics for the activities of the two substrates supports the latter suggestion. Enzyme purification studies will be necessary to resolve this question.

2',4',4,6'-Tetrahydroxychalcone and the 2',4',6'-tri-hydroxychalcone were both active as substrates for CHI in elicited cactus cultures; the 2',4,4'-trihydroxychalcone was not converted to the flavanone. Maximum CHI activity was detected 24 hr post-elicitation with an approximately 10-fold increase for the tetrahydroxychalcone substrate. The trihydroxychalcone substrate supported an approximately 10-fold lower level of activity than the alternative substrate, with maximum activity being recorded at 7 hr. The flavanone product naringenin was identified from the tetrahydroxychalcone substrate assay by its UV spectrum and by TLC co-migration with a standard. The different kinetics of induction with the trihydroxy- and tetrahydroxychalcone provide support for the presence in this cell culture of two CHI enzymes with different substrate specificity.

If aurone synthesis involves a peroxidase-mediated conversion of the chalcone as suggested by Wong [10], the high levels of KCN added in the CHI assay would be expected to inhibit the peroxidase; this would account for the conversion of chalcone to flavanone rather than chalcone to aurone, or other oxidation products, in the crude extracts used for CHI. CHI would not be needed for aurone synthesis if the chalcone were the substrate. Further studies are now needed to determine the precursor for aurone synthesis and the role, if any, of CHI.

There have been no previous reports of enzymes in the flavonoid pathway being induced in members of the Cactaceae; however, the level and rate of induction reported here are similar to those reported previously in relation to elicitor-induced isoflavonoid synthesis in bean and alfalfa cultures [11]. It is, therefore, likely that induced defence responses in the Cactaceae involve activation of flavonoid biosynthetic genes as observed in the Leguminosae [12]. The *C. senilis* system is ideal for
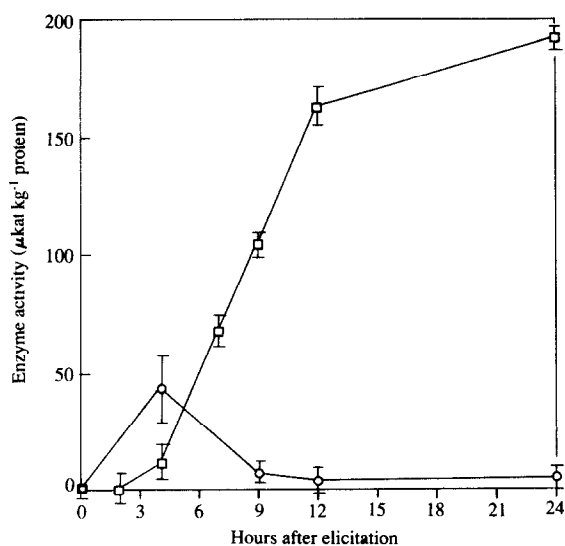
Fig. 3. Changes in extractable PAL activity in *C. senilis* cells in response to chitin (□—□) or in water treated controls (O—O).
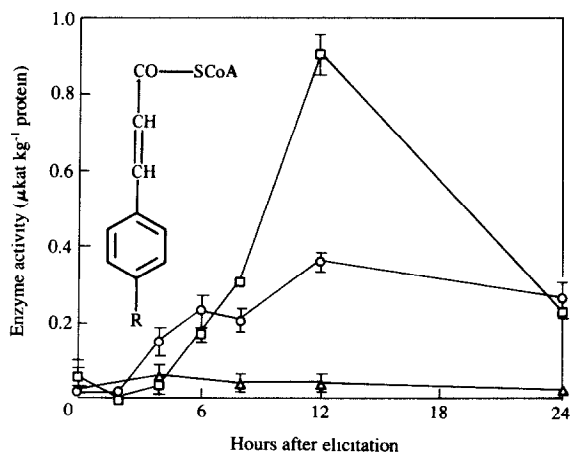

Fig. 4. Changes in extractable CHS activity in *C. senilis* cells in response to chitin with two substrates. □—□, elicited cells with 4-coumaroyl CoA (R = OH); O—O, elicited cells with cinnamoyl CoA (R = H); △—△, water treated controls.
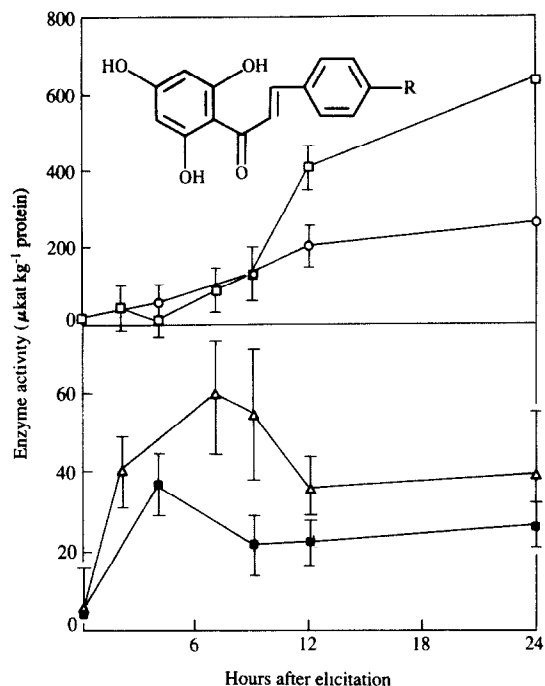

Fig. 5. Changes in extractable CHI activity in *C. senilis* cells in response to chitin with two substrates. □—□, elicited cells with tetrahydroxychalcone (R = OH); O—O, water treated controls. △—△, Elicited cells with trihydroxychalcone (R = H); ■—■, water treated controls.

further studies of the molecular biology of environmentally modulated gene regulation in a desert-adapted species.

EXPERIMENTAL

Plant material. Callus cultures of *Cephalocereus senilis* were initiated from inner stem tissue of greenhouse-grown plants and cultured on a medium containing MS minimal organic salts [13], 3% (w/v) sucrose, 0.2% (w/v) gelrite, 4.5 μM 2,4-dichlorophenoxyacetic acid, 4.4 μM N-6 benzyladenine, with the pH adjusted to 5.7. Suspension cells were initiated from callus material and cultured on a gyratory shaker (118 rpm) at 25° in the dark in the same medium without gelrite. Cells were sub-

cultured every fourth day by mixing 40 ml of suspension culture with 125 ml of fresh medium.

Chemicals and substrates. All substrates and reference compounds were obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) except L-[2,6-^{3}H]phenylalanine (specific activity 2.11 TBq mmol^{-1}) and [2-^{14}C]malonyl CoA (specific activity 2.186 Bq mmol^{-1}) (Amersham, Arlington Heights, IL).

p-Coumaroyl coenzyme A was previously prepared as described in [14] and cinnamoyl coenzyme A was synthesized as described in ref. [15]. The N-hydroxysuccinimide ester of cinnamic acid was prepared by slowly adding dicyclohexyl carbodiimide (16.96 mmol) to a mixt. of cinnamic acid (15.9 mmol) and N-hydroxysuccinimide (15.9 mmol). The succinimide ester was purified by recrystallization, and was then exchanged with coenzyme A lithium salt to yield cinnamoyl CoA. The cinnamoyl CoA was sepd from starting material on a Sephadex G-10 column eluted with 50 mM HCO$_2$H. The yield of cinnamoyl CoA (λ_{max} 262; log ε = 4.32, 310 nm) was 4.8%.

The 2',4',6'-trihydroxychalcone was prepd by mixing benzaldehyde (3.9 mmol) with 2',4',6'-trihydroxyacetophenone monohydrate (3.9 mmol) in 5 ml of EtOH; the soln was purged with N$_2$ at room temp. [16]. 50%(w/v) KOH soln (4 ml) was added dropwise to the soln. After purging overnight with N$_2$ the soln was acidified with 6 M HCl; the acidic suspension was dissolved in Et$_2$O and partitioned against NaHSO$_3$-satd H$_2$. The Et$_2$O layer was concd *in vacuo*, and the concentrate was applied to a silica gel-60 column which was then eluted with petrol-EtOAc (1 : 1) to remove unreacted starting materials. The chalcone was eluted from the column with MeOH. The 2',4',4,6'-tetrahydroxychalcone was prepared by the same procedure except benzaldehyde was replaced with 4-hydroxybenzaldehyde. Products were identified by UV spectroscopy; in the presence of

base, the product corresponds to the chalcone and in an acidic solution the product is the flavanone. The spectral findings were in agreement with published results [17]: for the synthetic 2',4',6'-trihydroxychalcone: UV λ_{max}^{NaOMe} 326; (HCl) 298, 326sh and for the 2',4',4,6'-tetrahydroxychalcone: UV λ_{max}^{NaOMe} 325; (HCl), 290, 325.

Elicitation of cell suspensions. Suspension cultures (4 × 165 ml) were combined and aliquoted into 15 ml samples and 30 ml of fr. medium was added. Cells were elicited 24 hr after subculturing with 5 ml of sterile chitin suspension (8 mg chitin ml^{-1} H$_2$O). Samples for labelling experiments were treated with 50 μl of sterile L-[2,6-^{3}H]phenylalanine (2.11 TBq mmol^{-1}, 0.88 nM). Unelicited (control) cells were treated with 5 ml sterile H$_2$O instead of the chitin suspension.

Phenolic analysis. Cells were harvested at timed intervals and extracted by a method adapted from the procedure of Elkind *et al.* [18]. The medium was decanted after centrifugation at *ca* 7000 *g* for 5 min at room temp.; to the cell precipitate 35 ml of chilled McCN were added. The suspension was sonicated for 10 min, and after centrifugation for 5 min, the residue was re-extracted in 50% (v/v) aq. MeCN (35 ml). The combined extracts were evapd to dryness *in vacuo*. The concentrate was redissolved in 2.5 ml 0.01 M HCl, and the soln acidified with 10 drops 1 M HCl; the acidic soln was applied to a C-18 Sep Pak cartridge (Waters). After washing the cartridge with 2 ml 0.01 M HCl, the phenolic fraction was recovered by eluting the SepPak with 2 ml H$_2$O-MeCN (3:2).

Sample injections of the phenolic fraction (50 μl each) were analysed by HPLC using a 250 ×4.6 mm Econosil (5 μ particle size) reverse phase C-18 column (Alltech, Deerfield, IL) eluted at 0.8 ml min^{-1} with a five-step gradient starting with 20% A, 20–35% (60 min), 35–100% A (1 min), hold at 100% A (9 min), 100–20% A (10 min). Solvent A was MeCN while solvent B was H$_2$O adjusted to pH 2.6 with conc H$_3$PO$_4$. The eluant was monitored by UV absorbance at 280 nm and for radioactivity assay with a Flow-One Beta HPLC radioactive flow detector (Radiomatic Instruments and Chemical Co., Inc., New Haven, CT). The radioactive aurone peak was identified by co-migration with a standard sample of the unlabelled aurone. A quench curve with 25% solvent A, spiked with tritium labelled phenylalanine, showed optimal counts at a 4:1 cocktail: effluent ratio with monoflow 5 scintillation fluid (National Diagnostics, Manville, N.J.). Aliquots of [^{3}H]phenylalanine were injected into the HPLC system to calculate the total amount of radioactivity (CPM) administered to the cultures.

Enzyme assays. After incubation with chitin, at timed intervals the cell suspension was filtered through Whatman No. 1 paper under vacuum and the collected cells were frozen in liquid N$_2$. The frozen cells were extracted and assayed directly for PAL and CHI activity by previously published spectroscopic assays [15]. PAL activity was also determined by measuring the incorporation of L-[U-^{14}C]phenylalanine into cinnamic acid as previously described [14]. The products of the CHI reaction were extracted with EtOAc and their UV absorbance were recorded in MeOH.

The conditions for the assay of CHS by measurement of the incorporation of [2-^{14}C]malonyl CoA into the flavanone have been described previously [19]. Reaction products were separ-

ated by TLC on aluminium-backed silica gel plates (Merck, Darmstadt) in CHCl$_3$-EtOH (3:1), and the flavanone product identified by co-migration with a standard. Quantitation of the malonyl CoA incorporation involved scrapping off the spot of interest and counting the radioactivity of the collected material.

Protein concentrations of enzyme extracts were determined by the Bio-Rad dye-binding reagent as detailed by the manufacturer using BSA as a standard [20].

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