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Stress Responses in Alfalfa (*Medicago sativa* L.)

XIV. Changes in the Levels of Phenylpropanoid Pathway Intermediates in Relation to Regulation of L-Phenylalanine Ammonia-Lyase in Elicitor-Treated Cell-Suspension Cultures

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We have used high-resolution gas chromatography to determine the levels of *trans*-cinnamic acid (CA) and *trans*-4-coumaric acid (4CA) in alfalfa (*Medicago sativa* L.) cell-suspension cultures to address the role of these phenylpropanoid pathway intermediates as potential negative regulators of phenylalanine ammonia-lyase (PAL) in vivo. Exogenous addition of CA to elicitor-treated cultures resulted in rapid increases in endogenous CA, 4CA, and CA-conjugate levels associated with inhibition of the appearance of PAL transcripts. Treatment of elicited cultures with α-aminooxy-β-phenylpropionic acid (AOPP), a potent and specific inhibitor of PAL activity in vivo, resulted in reductions of CA and 4CA, with concomitant increases in PAL transcripts and extractable enzyme activity. In contrast, treatment with tetcyclacis, an inhibitor of CA 4-hydroxylase, resulted in increased CA and CA-conjugate levels, decreased 4CA levels, and decreased PAL transcript levels and enzyme activity. In tetcyclasis-treated cells, the inhibition of PAL transcript appearance preceded the increase in the levels of free CA and its conjugates. In elicited cells in which the phenylpropanoid pathway was not perturbed by metabolic inhibitors, PAL transcripts accumulated rapidly and transiently, beginning to decline by 2 h postelicitation. Changes in levels of total free or conjugated CA or 4CA did not consistently correlate with these changes in transcript levels. We propose that regulation of PAL transcript levels by endogenous phenylpropanoid pathway intermediates could involve compartmentalized pools that may exist because of the microsomal localization of cinnamic acid 4-hydroxylase.

Leguminous plants respond to infection by fungal pathogens, or elicitor molecules from the cell walls of these pathogens, by synthesizing antimicrobial phytoalexins and wall-bound phenolics derived from L-Phe (Fig. 1). PAL catalyzes the first committed step in thebiosynthesis of thesedefense-related phenylpropanoids, the conversion of Phe into CA. CHS catalyzes the conversion of 4-coumaroyl Co-A into 2′,4,4′-trihydroxychalcone, the first branch point into the production of flavonoids and isoflavonoids. The phytoalexin defense response involves the rapid transcriptional activation of genes encoding a number of phytoalexin biosynthetic enzymes, including PAL and CHS (Hahlbrock and Scheel, 1989; Dixon and Harrison, 1991; Gowri et al., 1991; Paiva et al., 1991).

A large body of evidence suggests that phenylpropanoid pathway intermediates can regulate expression of the pathway at the posttranslational and transcriptional levels. Exposure of elicitor-treated bean (*Phaseolus vulgaris* L.) cell-suspension cultures to exogenous CA leads to a rapid loss of induced PAL enzyme activity (Dixon et al., 1980; Bolwell et al., 1986, 1988). A nondialyzable factor from CA-treated bean cultures stimulates removal of PAL activity from enzyme extracts in vitro; this effect requires the presence of CA (Bolwell et al., 1986), and the CA-mediated appearance of the factor appears to require protein synthesis (Mavandad, 1990). In potato tuber discs, bean cell-suspension cultures, and sunflower hypocotyls, the postinduction decline in PAL activity is blocked by inhibitors of RNA and/or protein synthesis in a manner consistent with a requirement for a second regulatory protein affecting PAL removal (Lamb, 1979; Dixon et al., 1980; Jorrin et al., 1990). Addition of CA to elicitor-treated bean cultures inhibits the appearance of PAL transcripts and a number of polypeptides translated in vitro from polysomal mRNA, although several polypeptides are specifically induced by CA treatment (Bolwell et al., 1988; Mavandad et al., 1990). Conversely, treatment of elicitor-induced bean cultures with AOPP, a potent and specific inhibitor of PAL activity in vivo (Amrhein and Gödeke, 1977), results in increased accumulation of PAL transcripts and enzyme activity (Bolwell et al., 1988; Mavandad et al., 1990). Following exogenous application of CA to elicited bean cultures, CHS transcript levels rapidly decline, and the increase in CHS enzyme activity is arrested, whereas treatment with AOPP results in increased production of CHS transcripts (Bolwell et al., 1988). Overexpression of a bean PAL gene in transgenic tobacco results in an overall decrease in PAL enzyme activity and phenolic levels, which is consistent with CA production being a down-regulatory signal (Elkind et al., 1990).

We have chosen cultured alfalfa cells as a model system in which to study the potential regulation of the phenylpropanoid pathway by early pathway intermediates. Studies with a bean *chs* promoter-chloramphenicol acetyltransferase con-

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Abbreviations: AOPP, L-α-aminooxy-β-phenylpropionic acid; β-ME, β-mercaptoethanol; BSTFA, bis(trimethylsilyl)trifluoroacetamide; CA, cinnamic acid; CA4H, cinnamic acid 4-hydroxylase (EC 1.14.13.11); 4CA, *trans*-4-coumaric acid; CHS, chalcone synthase; PAL, L-phenylalanine ammonia-lyase (EC 4.3.1.5); SSC, saline sodium citrate; THF, tetrahydrofuran; TMSCl, trimethlysilyl chloride; t_R, retention time.

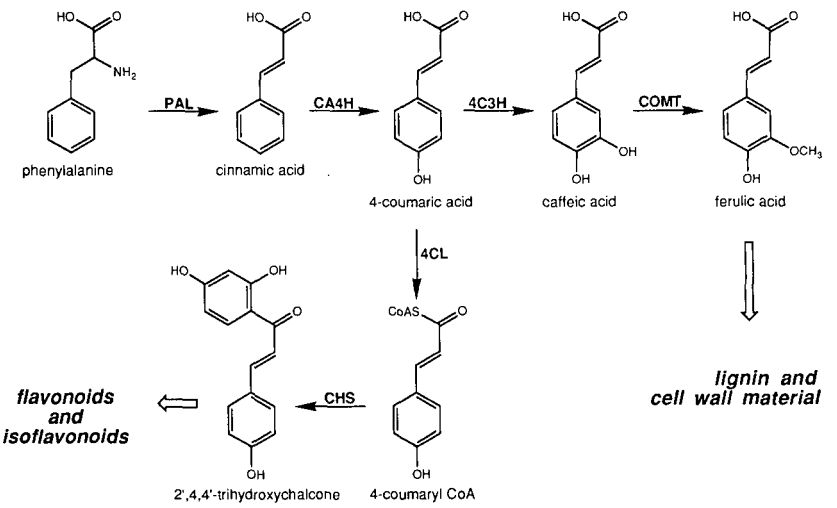

Figure 1. The phenylpropanoid biosynthetic pathway. The enzymes are PAL, CA4H, 4-coumarate 3-hydroxylase (4C3H), caffeic acid 3-*O*-methyltransferase (COMT), and 4-coumarate: CoA ligase (4CL).

struct electroporated into alfalfa protoplasts, which were then treated with fungal elicitor, have shown that exogenously applied CA (5–100 μM) weakly stimulates elicitor-induced chloramphenicol acetyltransferase expression from the bean *chs* promoter, whereas higher concentrations (>100 μM) severely reduce expression (Loake et al., 1991). In contrast, 4CA stimulates expression from the *chs* promoter up to 4.5-fold at 500 μM. Results of these studies suggest that specific *cis*-elements in the *chs* promoter may be involved in regulation by phenylpropanoid pathway intermediates (Loake et al., 1991) and that alfalfa cells contain the necessary *trans*-factors for the mediation of these effects.

Despite this large body of indirect evidence, the model in which CA or 4CA acts as a natural regulator of flux through the phenylpropanoid pathway in vivo is difficult to test directly. Relatively high concentrations (1 mM) of exogenously added CA are required for optimal inhibition of the induction of PAL activity in plant cells. Such concentrations could be cytotoxic (Friend, 1979), resulting in general inhibition of transcription, although this has been discounted in bean cells where exogenously applied CA at 1 mM does not inhibit the levels of a constitutively expressed transcript, H1 (Mavandad et al., 1990), or in alfalfa protoplasts where it does not appear to significantly affect cell viability (Loake et al., 1991). Alternatively, some contaminant in the CA sample could be the inhibitory agent. Furthermore, exogenous application of CA results in its conversion into many metabolites (Edwards et al., 1990), and the use of PAL inhibitors may result in reductions in the levels of many phenylpropanoid-derived compounds. Most important, the levels of free CA and its biosynthetic derivatives have not been determined in most of the previous studies that have suggested regulation by these compounds, and the methods that have been used in several studies have been found to be unreliable (Orr et al., 1993).

We have designed a high-resolution GC protocol that allows for the unequivocal determination of endogenous free CA and 4CA levels in alfalfa cells (Orr et al., 1993). We now report correlations between the levels of endogenous phenylpropanoid pathway intermediates, PAL transcript levels, and PAL enzymic activity in cells exposed to a number of different experimental manipulations of the flux through the phenylpropanoid pathway. Our data fail to show a direct relation between the levels of the total CA pool (free or conjugated) and changes in PAL transcript levels, although changes in endogenous CA pools brought about by inhibition of PAL or CA4H activities in vivo affect PAL transcripts in a manner consistent with a regulatory role for CA or a close derivative.

MATERIALS AND METHODS

Materials

[α-^{32}P]dATP was obtained from New England Nuclear (Boston, MA). Cinnamic, hydrocinnamic, 4-coumaric, caffeic, and ferulic acids, BSTFA, TMSCl, and THF-d_8 (99.5 atom % deuterium) were obtained from Aldrich (Milwaukee, WI). AOPP was purchased from Cambridge Research Biochemicals (Wilmington, DE), and tetcyclacis was a generous gift from Barbara Moore, BASF (Research Triangle Park, NC).

Synthesis of *cis*-CA

trans-CA (2.5 g) was dissolved in THF (90 mL) and irradiated at room temperature for 1 h with UV light generated with a 200-W medium-pressure mercury lamp powered by a 200-W source and passed through a vicor sleeve filter (all purchased from Ace Glass, Vineland, NJ). The volume of THF was reduced to 2 mL at 35°C in vacuo, and *cis*-CA was resolved from *trans*-CA and purified by preparative HPLC using an octadecyl silane column (250 × 22.5 mm with 10-μm packing) eluted at 10 mL min^{-1} with CH_3CN:H_2O:HCO_2H (34.7:64.3:1.0, v/v/v) (t_R [*cis*-, *trans*-CA] = 18, 22 min, respectively).

To confirm the progression of the reaction and identity of the product, *trans*-CA (9.8 mg) and THF-d_8 (0.5 mL) were placed in an NMR tube, irradiated as above for 15 min, and an NMR spectrum was obtained with a Varian (Palo Alto, CA) XL 300 MHz NMR spectrometer. The appearance of two new vinyl resonances at 5.90 and 6.84 ppm (J = 12.9 Hz)

confirmed the isomerization of the *trans*-carbon-carbon double bond, and the absence of changes in any other resonances confirmed the formation of one product.

DNA Probes

A cDNA complementary to an alfalfa PAL (471-bp *Hin*dIII internal coding region fragment [positions 831–1301]) (Gowri et al., 1991), a 1.6-kb full-length alfalfa CHS cDNA (K. Dalkin, H. Junghans, and R.A. Dixon, unpublished results), and the cDNA clone for the constitutively expressed H1 transcript from bean (Lawton and Lamb, 1987) were used in northern blot analyses as described below. The PAL and CHS probes do not discriminate between different members of the alfalfa *pal* and *chs* multigene families.

Treatment of Plant Cell Cultures

Alfalfa cell-suspension cultures were initiated and maintained as described previously (Dalkin et al., 1990). Fungal elicitor was prepared by autoclaving partially purified cell walls of *Colletotrichum lindemuthianum* as described by Dixon and Lamb (1979), and yeast elicitor was prepared as described by Schumacher et al. (1987). Elicitors and chemicals were added to cultures as filter-sterilized solutions. Dark-grown suspension-cultured cells were treated 5 d after subculture with elicitor at a final concentration of 50 μg of Glc equivalents mL^{-1} of medium, and control cultures were treated with an equivalent volume of water. *trans*- and *cis*-CA were added as acetone solutions so that the final phenylpropanoid concentrations were 1.0 or 0.1 mM and the final acetone concentrations were 0.25 or 0.025% (v/v), respectively.

AOPP, tetcyclacis, and 4CA were added so that the final concentrations were 50, 50, and 500 μM, respectively. AOPP was added as an aqueous solution, 4CA was added in Tris-HCl buffer (pH 7), resulting in a final Tris concentration of 160 μM, and tetcyclacis was added in acetone:water 1:1 (v/v) with a final acetone concentration of 0.12% (v/v). Duplicate cell batches were harvested at timed intervals by vacuum filtration through 20-μm nylon mesh, washed with 300 mL of water, frozen with liquid nitrogen, and stored at −80°C until required for analysis.

Isolation and Analysis of RNA

Total RNA was isolated by the method of Chomczynski and Sacchi (1987) with the following modification: Before reprecipitation of RNA with isopropanol (see table I in Chomczynski and Sacci, 1987), samples dissolved in solution "d" were placed on ice for 15 min and then clarified by centrifugation. The supernatant was then removed and processed as described.

RNA was fractionated by electrophoresis through formaldehyde-containing agarose gels as described by Maniatis et al. (1989), transferred to nitrocellulose membranes as described by Kroczek and Siebert (1990), and fixed with UV irradiation from a Stratagene UV Stratalinker 1800. Prehybridization was performed in buffer containing 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5× Denhardt's reagent, 0.5% (w/v) SDS, and 100 μg of sheared denatured salmon sperm DNA mL^{-1}, at 42°C for 16 h. Denatured probe was added (5×10^7 cpm mL^{-1} for PAL and CHS and 5×10^6 cpm mL^{-1} for H1), and hybridization was performed for 16 h. Blots were washed 3 × 20 min with 0.1× SSC, 0.1% SDS at 65°C.

Extraction and Assay of PAL Activity

PAL was extracted from cell cultures that had not been treated with AOPP as described by Bolwell et al. (1985b). AOPP-treated cultures (and the corresponding control cultures) were extracted for PAL as follows, so as to remove inhibitory AOPP from the crude enzyme extract. Frozen cells (500 mg) were extracted with a blade homogenizer in 1 mL of 50 mM Tris-HCl, 0.1% (v/v) β-ME (buffer A) saturated with L-Phe, and incubated at room temperature for 1 h. After the addition of 40 mg of polyvinylpolypyrrolidone and centrifugation for 10 min, the supernatant (1 mL) was concentrated to 50 to 100 μL with a Centricon-30 Microconcentrator (Amicon, Danvers, MA), combined with 2 mL of buffer A, and reconcentrated, and the last cycle was repeated twice. The final retentate was assayed for PAL enzyme activity. PAL enzyme assays were performed using a previously described spectrophotometric procedure (Bolwell et al., 1985a). Protein concentrations were determined using the Bio-Rad (Richmond, CA) dye-binding reagent.

Extraction and Determination of Medicarpin, CAs, and CA Conjugates

Frozen cells (1.0 g) that had been treated with 1.0 mM CA were combined with 100 nmol of hydrocinnamic acid (internal standard) and extracted with a blade homogenizer in 2 mL of ice-cold phosphate buffer (0.02 M, pH 8.1) containing 1% (v/v) β-ME. Insoluble material was removed by centrifugation at 4°C. The supernatant was acidified with 40 μL of 4 N HCl and extracted with 2 mL of ethyl acetate. The phases were separated by centrifugation, and the ethyl acetate layer was removed, cooled to −40°C for 1 h, separated from the ice that formed, dried with a stream of N_2 at 45°C in a Pierce Model 18800 evaporating unit (Rockford, IL), and used for determination of free CAs.

Frozen cells not treated with CA (500 mg) were spiked with hydrocinnamic acid (100 nmol) and extracted with a blade homogenizer in 3 mL of ice-cold ethyl acetate: HCl-KCl buffer (0.1 M in Cl^-, pH 2.0):β-ME (2:1:0.001, v/v/v). The ethyl acetate layer was removed and dried as described above. Part of the organic extract (20%) was used for medicarpin determinations, and the remainder was used for determination of free CA.

Cell extract residues were dissolved in 200 mL of methanol and analyzed for medicarpin by reversed phase HPLC (Koster et al., 1983). Identification of medicarpin was accomplished by cochromatography and by comparing its UV absorption spectrum with that of an authentic standard. Quantification of medicarpin was achieved by calibration with the authentic standard.

For determination of free CAs, organic cell extract residues were combined with 100 μL of dry, distilled pyridine, and 100 μL of BSTFA containing TMSCl (1%, v/v) in capped

autosampler vials and heated to 80°C for 15 min, and 1 μL of each sample was then analyzed by GC on a Supelco (Bellefonte, PA) SPB-5 capillary column. Quantification of cinnamic, hydrocinnamic (internal standard), 4-coumaric, caffeic, and ferulic acids was achieved by calibration with authentic standards and by normalization with the internal standard peak. To confirm identities of the different silylated CAs, coinjections with authentic silylated standards were performed. The silyl derivatives were also hydrolyzed with dilute acid, and the resultant carboxylic acids were methylated with diazomethane and analyzed again by GC with coinjection with authentic methyl cinnamate and methyl 4-coumarate. Finally, GC-MS was used to confirm the nature and purity of the CA and 4CA peaks in separated cell extracts (Orr et al., 1993).

For determination of CA conjugates, frozen tissues were combined with 100 nmol of chlorogenic acid (internal standard) and 4 mL of ice-cold acetonitrile and sonicated with a Kontes (Vineland, NJ) Micro Ultrasonic Cell Disrupter at 100 W for 30 s before centrifugation at 8000*g* for 10 min at 4°C. The pellet was combined with 4 mL of 50% aqueous acetonitrile (prechilled to 4°C), resonicated, and centrifuged as above. The extracts were combined, and the acetonitrile was removed under a stream of N_2 at 45°C. The resultant aqueous extracts were adjusted to pH 2.0 with 1 N HCl, loaded onto an ODS Sep-Pak (Waters, Milford, MA) 1- × 1-cm column, washed with 3.5 mL of 0.01 N HCl, and eluted with 3.5 mL of acetonitrile:water (2:3, v/v). The eluate was dried under a stream of N_2 at 45°C. The residue was dissolved in 200 μL of acetonitrile:water (2:3, v/v) and clarified by centrifugation, and 20-μL aliquots were analyzed by HPLC using an octadecyl silane column (250 × 4.6 mm with 5-μm packing) eluted at 1 mL min^{-1} with a four-step gradient of increasing solvent B in solvent A of 5% B (5 min), 5 to 10% B (5 min), 10 to 35% B (15 min), 35 to 40% B (5 min). Solvent A was water:formic acid (98:2, v/v), and solvent B was acetonitrile:formic acid (98:2, v/v). The eluant was monitored for UV A_{271}; quantification was achieved by calibration with CA and by normalization to the internal standard peak. Confirmation of identity was made following collection of the putative CA conjugate peaks as follows. Solvent was removed under a stream of N_2 at 45°C, the residue was hydrolyzed at 90°C for 1 h with 2 N HCl and extracted with ethyl acetate (3 × 1 mL), and the combined organic layers were dried and silylated with 100 μL of BSTFA containing TMSCl (1%, v/v) and 100 μL of pyridine at 80°C for 15 min and then analyzed for the presence of trimethylsilyl-CA by capillary GC.

RESULTS

Down-Regulation of PAL Expression by Exogenously Applied CA Derivatives in Elicited Cell-Suspension Cultures

As previously reported for bean (Bolwell et al., 1988; Mavandad et al., 1990), exposure of elicited alfalfa cell-suspension cultures to 1 mM *trans*-CA resulted in a complete suppression of appearance of PAL transcripts 2.5 h postelicitation (Fig. 2), with partial inhibition resulting from exposure to 0.1 mM CA. Because of the high concentrations of *trans*-CA required to inhibit PAL transcript appearance, it is possible that some contaminant of the *trans*-CA preparation could be the active factor. However, *cis*-CA, the most likely potential contaminant, inhibited PAL transcript appearance in an identical manner with *trans*-CA at 1 mM and was probably less inhibitory at 0.1 mM (based on PAL:H1 ratio). If *cis*-CA were the active component, the 0.1 mM treatment with this compound would be expected to be more inhibitory than 0.1 mM *trans*-CA, which was not the case (Fig. 2). None of the treatments strongly affected the level of H1 transcripts. H1 is expressed constitutively in bean cells and exhibits a high rate of transcription (and therefore turnover) compared to PAL (Mavandad et al., 1990). Therefore, it serves as a good control for inhibitory effects of applied chemicals on general transcription and as a reference for correcting gel loading.

Because CA is rapidly absorbed and metabolized in bean cultures (Edwards et al., 1990), the observed down-regulation of PAL transcript appearance by exogenously applied CA could be mediated by a metabolite of CA rather than CA itself. To clarify this situation with respect to alfalfa cells, the levels of free cinnamic, *trans*-4-coumaric, ferulic, and caffeic acids were determined in cell-suspension cultures treated with 1.0 mM CA 4.5 h after treatment with yeast elicitor. Figure 3A shows an HPLC trace of an extract from an alfalfa cell suspension after exposure to yeast elicitor for 4.5 h. Addition of 1.0 mM CA to the culture resulted in accumulation of CA, 4CA, and two CA conjugates of retention times of 23.1 and 24.3 min (Fig. 3B). The identity of the conjugates was confirmed by hydrolysis and analysis of the released CA by GLC (data not shown). HPLC does not resolve CA from a major cochromatographing peak (compare the relative intensities of the CA peaks in Fig. 3, A and C). GC analysis confirmed that free CA and 4CA were present in low

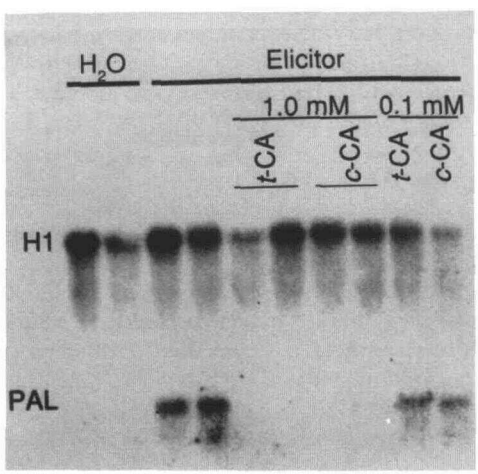

Figure 2. Northern blot analysis of PAL and H1 transcripts in alfalfa cell-suspension cultures 2.5 h after exposure to yeast elicitor or H_2O (control) in the presence or absence of *trans*-CA (*t*-CA) or *cis*-CA (*c*-CA). Cultures not treated with phenylpropanoids received acetone at a final concentration of 0.25% (v/v). Samples are duplicated in lanes 1 to 8.

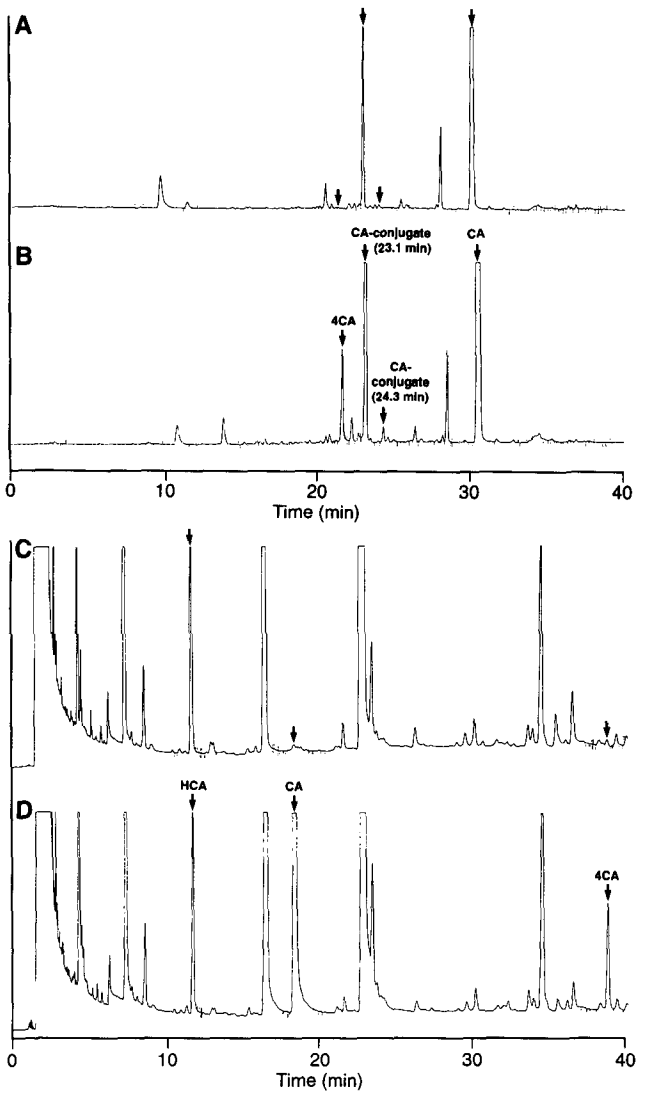

Figure 3. HPLC (A and B) and GC (C and D) analysis of CA derivatives in extracts from alfalfa cell-suspension cultures. Cells were harvested 4.5 h after exposure to yeast elicitor (A and C) or 3.5 h after addition of 1 mM CA to 4.5 h-elicited cultures (B and D). HCA, Hydrocinnamic acid (internal standard). See "Materials and Methods" for details of HPLC and GC protocols.

amounts in elicited cells (Fig. 3C), and their levels increased dramatically when the cells were fed CA (Fig. 3D). By 1.5 h after addition, the level of CA associated with the cells increased from approximately 30 μM to 1.2 mM, a factor of 40, and then slowly decreased during the next 4 h (Fig. 4). Endogenous 4CA levels increased from approximately 9 to 56 μM, indicating flux through the CA4H reaction, whereas no consistent changes were observed in the levels of ferulic and caffeic acids. Thus, down-regulation of PAL caused by addition of CA correlates with increased endogenous levels of CA, 4CA, or CA conjugates but not with ferulic or caffeic acid levels.

4CA has been shown to inhibit the appearance of PAL enzymic activity in elicited bean cells (Dixon et al., 1980) and

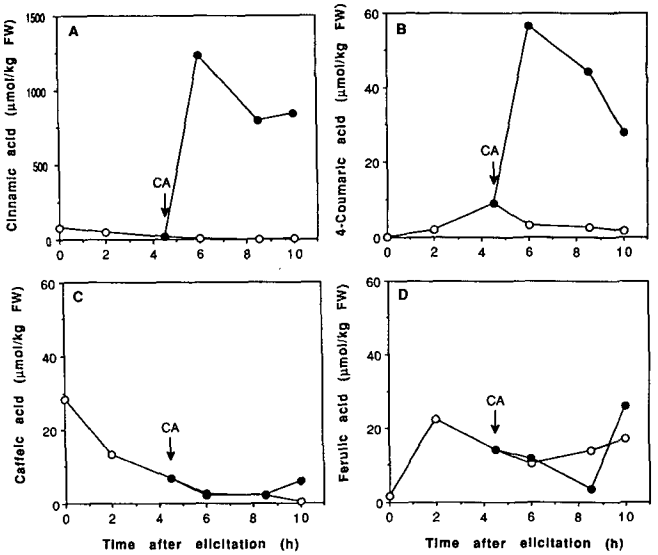

Figure 4. Levels of CA (A), 4CA (B), caffeic acid (C), and ferulic acid (D) in alfalfa cell-suspension cultures treated with yeast elicitor (50 μg of glucose equivalents mL^{-1}) (O) or in cultures treated with 1.0 mM cinnamic acid (●) 4.5 h after exposure to yeast elicitor. FW, Fresh weight.

to up-regulate expression of a bean CHS promoter in alfalfa protoplasts (Loake et al., 1991). To address further the possibility of 4CA as a regulator of the phenylpropanoid pathway at the transcriptional level, we measured the levels of PAL transcripts in alfalfa cells exposed to exogenously applied 4CA at the concentration that gave maximal up-regulation of the bean CHS promoter (Loake et al., 1991). The data in Figure 5 indicate that 4CA delays, but does not prevent, the appearance of elicitor-induced PAL transcripts in the alfalfa cell-suspension cultures. These results were confirmed in an independent experiment in which transcript

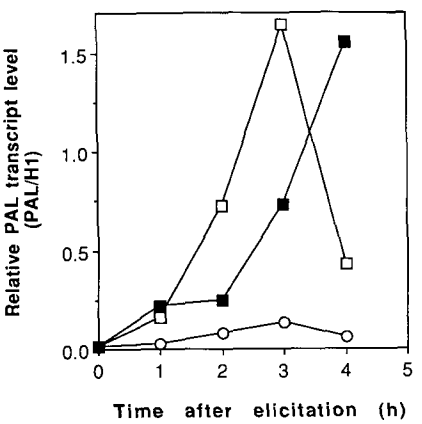

Figure 5. Changes in PAL transcript levels in unelicited alfalfa cell-suspension cultures (O), and in cultures exposed to yeast elicitor (50 μg of Glc equivalents mL^{-1}) plus (■) or minus (□) 4CA (final concentration 0.5 mM) added at the time of elicitation. PAL transcript levels are expressed relative to the levels of the constitutive transcript H1.

levels were measured up to 6 h postelicitation (data not shown).

Effects of Reducing Endogenous CA Levels on Phenylpropanoid Pathway Intermediates and Gene Transcript Levels

If high levels of CA, 4CA, or a related metabolite signal reduction of PAL mRNA and enzymic activity levels in vivo, decreasing the levels of these phenylpropanoids should result in a corresponding increase in PAL transcripts or activity. Previous studies have shown that treatment of elicited bean cultures with AOPP, a potent and specific inhibitor of PAL activity, and, therefore, CA production in vivo, results in increased accumulation of PAL transcripts and enzyme activity (Bolwell et al., 1988; Mavandad et al., 1990). However, levels of phenylpropanoid pathway intermediates were not measured in these studies, and no data are available concerning the effects of AOPP on PAL transcripts in alfalfa cultures, although the inhibitor has been shown to decrease the accumulation of medicarpin in these cells (Kessmann et al., 1990).

The levels of medicarpin, CA, 4CA, PAL transcripts and enzymic activity, and CHS transcripts were strongly induced in alfalfa cell-suspension cultures 4 h after treatment with fungal elicitor (Table I). Note that in this experiment there was a nearly 10-fold increase in the level of the free CA pool by 4 h postelicitation. Treatment of these cultures with AOPP at zero time caused 34, 91, and 75% reductions in the levels of medicarpin, free CA, and 4CA, respectively, and increased the levels of PAL transcripts, PAL enzymic activity, and CHS transcripts by 34, 14, and 80%, respectively. The level of PAL enzymic activity in AOPP-treated cultures may be an underestimate of the actual value because residual AOPP not removed while preparing the enzyme extracts could inhibit the conversion of Phe to CA in the PAL enzyme assay; PAL has a 3 orders of magnitude greater affinity for AOPP than for Phe (Amrhein and Gödeke, 1977).

In this experiment, only small increases in the levels of PAL enzymic activity and transcripts were associated with the predicted depression of CA and 4CA levels by AOPP. The AOPP treatment did, however, result in a significantly increased appearance of CHS transcripts, consistent with previous results in bean cells in which PAL transcripts and enzymic activity in elicited cells are increased by approximately 2-fold in the presence of AOPP (Bolwell et al., 1988). Having confirmed that AOPP behaved in alfalfa cells in a manner essentially similar to its effects on bean cells, we attempted to test further the model of pathway intermediate regulation by examining the effects of an agent that would be predicted to increase endogenous CA pools in vivo.

Table I. *Effects of AOPP (50 μM) on the levels of phenylpropanoid pathway intermediates, medicarpin, PAL, and CHS transcripts and extractable enzymic activity of PAL in elicitor-treated alfalfa cell-suspension cultures*

Phenylpropanoid Pathway Component	Time after Elicitation (h)[a]		
	0	4	
		(−) AOPP	(+) AOPP
CA (μmol kg^{-1} fresh wt)	2.5	23.2 (100)[b]	2.0 (8.6)
4CA (μmol kg^{-1} fresh wt)	13.5	35.0 (100)	8.8 (25.2)
Caffeic acid (μmol kg^{-1} fresh wt)	5.0	9.8 (100)	7.2 (73.2)
Medicarpin (μmol kg^{-1} fresh wt)	nd[c]	1.2 (100)	0.8 (65.9)
PAL mRNA/H1 mRNA (relative level)[d]	(4.6)	(100)	(134)
CHS mRNA/H1 mRNA (relative level)	nd	(100)	(180)
PAL enzyme activity (μkat kg^{-1} of protein)	32.7	439.0 (100)	499.0 (114)

[a] Cells were exposed to elicitor from the cell walls of *C. lindemuthianum* (final concentration 50 μg of glucose equivalents mL^{-1}). [b] Numbers in parentheses are the percentages compared to the (−) AOPP treatments. [c] nd, Not detected. [d] H1 is a constitutively expressed transcript that provides a control for loading and transfer efficiency in northern blots, and controls for effects of chemical treatments on general transcription, as discussed in text.

Effects of Increasing Endogenous CA Levels on Phenylpropanoid Pathway Intermediates and Gene Transcript Levels

In the experiment shown in Figures 6 and 7, alfalfa cell-suspension cultures treated with yeast elicitor alone exhibited no changes in the levels of free CA (in contrast to the data in Table I), although it should be noted that no measurements were made in the experiments shown in Figure 7 beyond 8 h postelicitation, and PAL transcripts had not significantly declined by this time (Fig. 6A). Also, no changes were observed in caffeic acid and H1 transcript levels, whereas the levels of medicarpin, 4CA, PAL mRNA, and PAL enzymic activity were all induced (Figs. 6 and 7). Concomitant treatment with elicitor and tetcyclacis (an inhibitor of CA4H activity in vivo [Rademacher et al., 1987]) caused a 4- to 5-fold increase in the level of CA between 2 and 4 h postelicitation (Fig. 7A) and inhibited 4CA and medicarpin accumulation (Fig. 7, B and D) but had no effect on caffeic acid levels (Fig. 7C). This treatment blocked accumulation of PAL transcripts and enzymic activity for up to 2 h postaddition and reduced their levels at later times (Fig. 6, A and C). Tetcyclacis had no significant effect on the levels of H1 transcripts (Fig. 6B).

Addition of tetcyclacis to cells 2 h after elicitation caused a 2- to 3-fold increase in CA levels and inhibited the accumulation of 4CA, medicarpin, and PAL enzymic activity but had no effect on caffeic acid levels. PAL transcript accumulation was reduced 2 h after tetcyclacis treatment but not at later times. Again, delayed tetcyclacis treatment had no effect on H1 transcript levels, indicating that the effects on PAL mRNA are not the result of a nonspecific inhibition of transcription.

These results further show that manipulation of CA levels in vivo has predictable effects on PAL transcript levels, consistent with the hypothesis that the endogenous level of CA or some derivative thereof can signal regulation of PAL mRNA and enzymic activity levels. However, if we assume the rapid uptake and effectiveness of tetcyclacis in blocking CA4H activity, the delayed accumulation of free CA shown

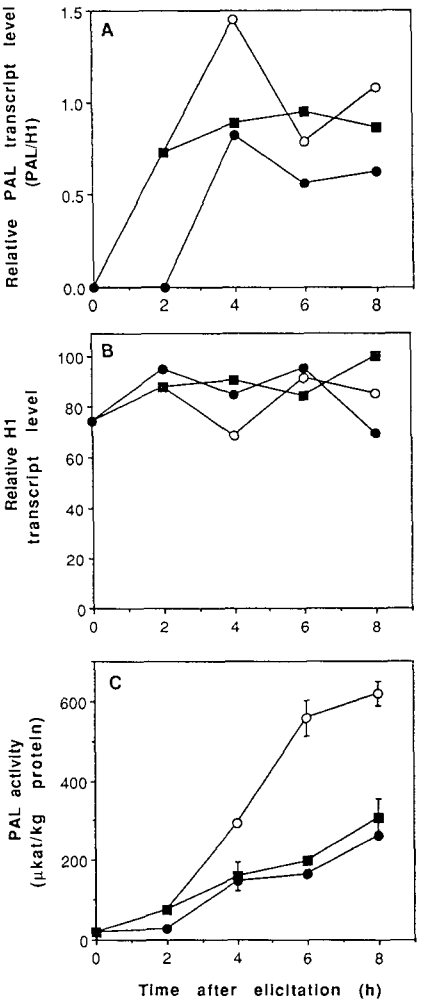

Figure 6. Levels of PAL mRNA (relative to H1) (A), H1 mRNA (B), and PAL enzymic activity (C) in alfalfa cell-suspension cultures treated with yeast elicitor (50 μg of Glc equivalents mL^{-1}) in the absence (O) or presence of tetcyclacis added at the same time as elicitor (●) or 2 h post elicitation (■). Further analyses of the same cell samples are shown in Figure 7. Bars indicate spread of duplicate determinations (extractions of separate cell batches).

in Figure 7A in relation to the inhibition of PAL transcript appearance would tend to rule out a role for the bulk pool of free CA as a regulatory signal. Therefore, we investigated whether accumulation of any conjugate of CA occurred during the inhibition of PAL transcript appearance and before the increase in free CA levels in the cells analyzed in Figures 6 and 7.

Two major CA conjugates were detected when exogenous CA was fed to alfalfa cells. These had HPLC retention times of 23.1 and 24.3 min, respectively (Fig. 3). Treatment of elicited cells with tetcyclacis resulted in increased levels of both conjugates (Fig. 7, E and F), indicating that prevention of the hydroxylation of CA leads to spillover into conjugation pathways. However, there was no apparent increase in the level of either conjugate by 2 h postelicitation, at which time the level of PAL transcripts had been reduced by the tetcyclacis treatment. Therefore, we conclude that it is unlikely that the observed increase in either of these conjugates plays a role in regulating PAL transcript levels.

Changes in Phenylpropanoid Pathway Intermediates in Response to Elicitor Alone

In the absence of tetcyclacis (i.e. in cells treated with elicitor alone), there was no significant increase in CA levels during the first 8 h postelicitation in the experiment shown in Figure 7A, whereas in the experiment shown in Table I, a 10-fold increase in CA levels was observed by 4 h postelicitation. A major difference between these two experiments was the use of elicitor from *C. lindemuthianum* for the AOPP experiment and yeast elicitor for the tetcyclacis experiment. Therefore, we compared, in a separate experiment, the effects of the two elicitors on the levels of free and conjugated CA and 4CA (Fig. 8).

Alfalfa cells treated with fungal elicitor responded differently from those treated with yeast elicitor. Treatment with yeast elicitor resulted in maximum levels of free CA and 4CA at 4 and 10 h postelicitation, respectively, whereas treatment with fungal elicitor resulted in an apparent increase in free CA levels at 10 h postelicitation and a decrease in free 4CA

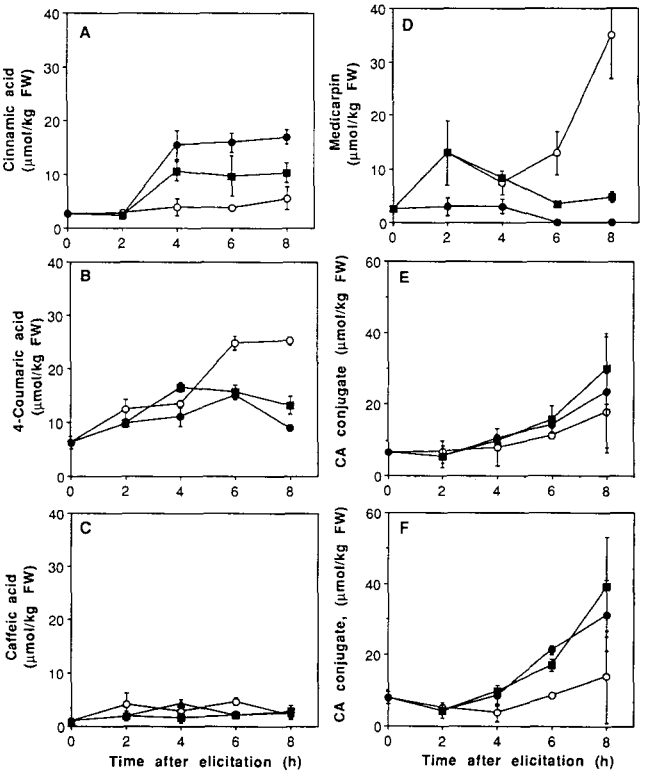

Figure 7. Levels of CA (A), 4CA (B), caffeic acid (C), medicarpin (D), CA conjugate t_R = 23.1 min (E), and CA conjugate t_R = 24.3 min (F) in alfalfa cell-suspension cultures treated with yeast elicitor (50 μg of Glc equivalents mL^{-1}) in the absence (O) or presence of tetcyclacis (50 μM) added at the same time as elicitor (●) or 2 h post elicitation (■). Bars indicate spread of duplicate determinations (extractions of separate cell batches). FW, Fresh weight.

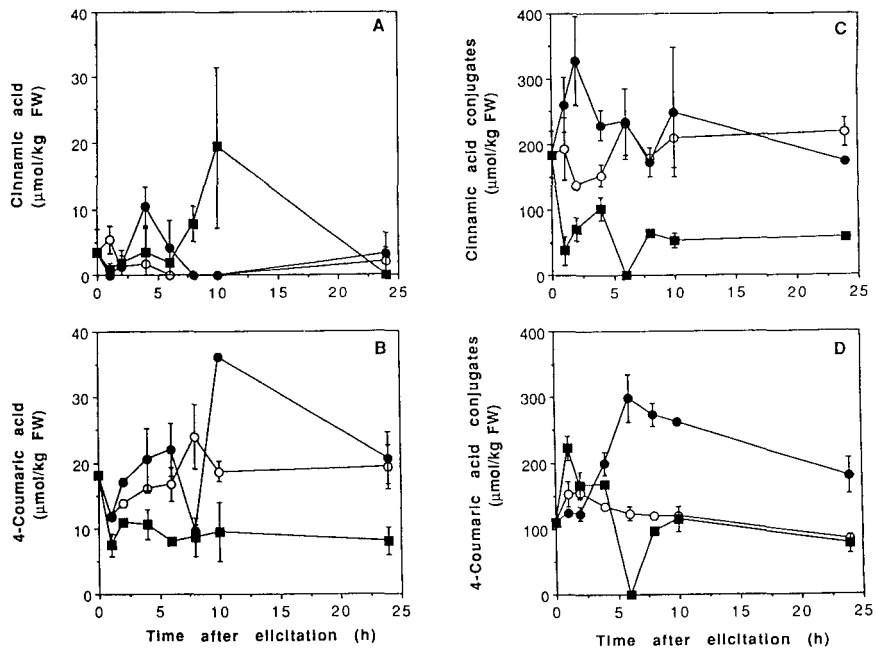

Figure 8. Levels of CA (A), 4CA (B), total CA conjugates (C), and total 4CA conjugates (D) in alfalfa cell-suspension cultures after treatment with water (control) (○), yeast elicitor (●), or fungal elicitor (■) (50 μg of Glc equivalents mL^{-1}). Bars indicate spread of duplicate determinations (extractions of separate cell batches). FW, Fresh weight.

levels (Fig. 8, A and B). Treatment with yeast elicitor resulted in maximum levels of CA and 4CA conjugates 2 and 6 h postelicitation, respectively (Fig. 8, C and D). Treatment with fungal elicitor resulted in decreased levels of conjugated CA, whereas levels of conjugated 4CA were slightly elevated at 2 h postelicitation. Thus, in contrast to the data shown in Figure 7, the levels of phenylpropanoid pathway intermediates in this experiment changed significantly during elicitation, but the extent and timing of the changes depended on the elicitor used. Note that the data in Figure 8 refer to total conjugate levels and do not distinguish between different potential conjugates of CA and 4CA.

The levels of the various CAs were measured further in a separate experiment in which we previously had determined striking transient changes in PAL transcripts, CHS transcripts, and PAL enzyme activity in response to yeast elicitor (Fig. 9). These cell-suspension cultures accumulated high levels of medicarpin, confirming that the phytoalexin biosynthetic pathway was induced (Fig. 9F). In contrast to the data shown in Figure 8, treatment with yeast elicitor had little effect on the already low CA pool size in the cells used in this experiment (Fig. 9C). There is, therefore, no consistent relationship between the bulk CA pool and the onset of the decline in elicitor-induced PAL transcripts. A rapid, transient, 4-fold increase in the level of 4CA was observed on elicitation, with only a slight increase in caffeic acid levels (Fig. 9, D and E). In contrast to the lack of change in the free CA pool, the levels of the two major conjugates of CA, especially the t_R = 24.3 min conjugate, increased on elicitation, although not until at least 2 h after exposure to elicitor (Fig. 9, G and H). This increase precedes the decrease in PAL enzyme activity, but not PAL transcript levels, in these cultures.

DISCUSSION

The previous studies that have led to the proposal of a role for CA and 4CA as potential modulators of the phenylpropanoid pathway at both transcriptional and posttranscriptional levels did not determine the endogenous levels of these compounds in relation to the changes they were proposed to be modulating. We had found it difficult to accurately and unambiguously determine the levels of endogenous free CA in legume cell cultures. Previous methods relied on HPLC analyses of crude cell extracts, but they appeared unreliable, at least in alfalfa cells, because CA was not resolved from a cochromatographing substance (Fig. 3A). We have now used a high-resolution capillary GC method that resolves CA and 4CA from all components of the plant cell extracts (Orr et al., 1993). Determinations of caffeic and ferulic acids by this method are sometimes hampered by cochromatographing material; the levels of these compounds were measured in each experiment in the present paper but are only reported when they were clearly resolved. To test the hypothesis that CA and its metabolites may act as endogenous regulators of PAL and CHS expression, we have monitored the changes in the levels of CA and some of its biosynthetic products in relation to changes in the levels of PAL transcripts and enzymic activity in alfalfa cell cultures under a variety of conditions.

If we assume no subcellular compartmentalization, the present data indicate overall concentrations of free CA and 4CA in alfalfa cells to be in the range of approximately 5 to 30 μM, whereas levels of total conjugated CA and 4CA were much higher. This is consistent with previous observations in bean (Bolwell et al., 1985b). Exogenously added CA or 4CA at 100 μM can cause significant down-regulation and up-regulation, respectively, of *chs* promoter expression in alfalfa protoplasts (Loake et al., 1991). The endogenous pool sizes of these compounds in alfalfa cells would, therefore, appear sufficient to regulate the expression of phenylpropanoid pathway biosynthetic genes, especially because it is unlikely that the concentration of each compound is equal throughout the cell.

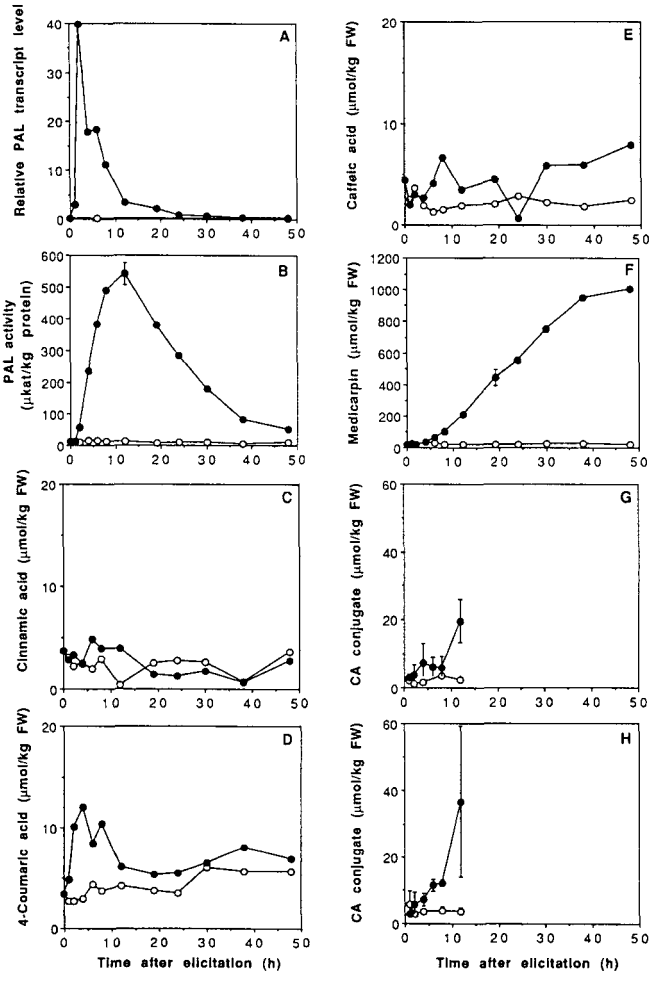

Figure 9. Levels of PAL mRNA (A), PAL enzymic activity (B), CA (C), 4CA (D), caffeic acid (E), medicarpin (F), CA conjugate t_R = 23.1 min (G), and CA conjugate t_R = 24.3 min (H) in alfalfa cell-suspension cultures after treatment with water (control) (○) or yeast elicitor (50 μg of Glc equivalents mL^{-1}) (●). Medicarpin levels are taken from Paiva et al. (1991), in which the same batch of cells was analyzed. Bars indicate spread of duplicate determinations (extractions of separate cell batches). FW, Fresh weight.

Feeding exogenous CA to the alfalfa cultures resulted in a striking increase in CA associated with the cells (all of which may not be internalized) and a significant (6-fold) increase in endogenous 4CA levels. Because feeding of CA did not affect the levels of caffeic and ferulic acids, it is unlikely that these later phenylpropanoid compounds are the mediators of the CA effect. This is supported by earlier data that indicated that exogenously supplied caffeic and ferulic acids were far less active inhibitors of PAL activity appearance in bean cell cultures than were CA or 4CA (Dixon et al., 1980). Also, our data rule out the possibility that contaminating *cis*-CA is responsible for the down-regulation of PAL by exogenously added *trans*-CA. Both isomers of CA appear equally effective in inhibiting PAL transcript appearance. In contrast, earlier studies have shown that *cis*-CA, unlike the *trans* isomer, is not inhibitory to PAL enzymic activity in vitro (Koukol and Conn, 1961; Engelsma, 1974).

Exogenously applied 4CA delays PAL transcript appearance by approximately 2 h in elicited alfalfa cells but does not inhibit the final transcript level obtained (Fig. 5). We do not know whether the recovery from this delay is due to subsequent metabolism of 4CA and cannot, therefore, unequivocally rule out a role for 4CA as a regulator of PAL transcription, although this is not consistent with the results of the tetcyclacis experiment (Figs. 6 and 7). The results of exogenous CA feeding experiments (Bolwell et al., 1986, 1988; Mavandad et al., 1990; Loake et al., 1991) and the superinduction phenomena with AOPP (Bolwell et al., 1988; Mavandad et al., 1990; Table I) do not rule out the possibility that 4CA could be a natural regulator of PAL expression at the enzyme level. This is supported by the kinetics of changes in endogenous 4CA levels observed in elicited cells in Figure 9D in relation to the corresponding changes in PAL enzyme activity (but not transcripts). In spite of the variation in changes in CA levels in the various experiments reported in this paper, the levels of CA plus 4CA increase in all cases between 4 and 10 h postelicitation. This increase could signal removal of active enzyme, a phenomenon known to be induced by exogenously added CA and dependent on protein synthesis (Dixon et al., 1980; Bolwell et al., 1986; Jorrin et al., 1990).

If free CA were to be an in vivo regulator of PAL transcription, the following would be predicted: (a) inhibiting the accumulation of CA in vivo would result in increased PAL expression (at the transcript and enzyme activity levels), (b) stimulating the accumulation of CA in vivo (either by exogenously feeding or artificially increasing endogenously formed pools) would result in decreased PAL expression, and (c) endogenous CA pools might increase before the decline phase in elicitor-induced PAL transcripts and enzyme activity. The data in this paper indicate that the first two criteria are met but that changes in endogenous CA levels on elicitation do not necessarily relate to changes in PAL transcripts and enzyme activity; indeed, strong down-regulation of elicitor-induced PAL transcripts and activity can occur with no significant change in the levels of free CA (Fig. 9). Furthermore, when CA4H activity is blocked by tetcyclacis (confirmed by inhibition of 4CA appearance and increased appearance of CA and CA conjugates), PAL transcript levels are affected before the detectable increase in the CA and CA conjugate pools (Figs. 6 and 7).

Two hypotheses accord with the above findings. First, the lack of a consistent increase in CA pool size at the time of PAL transcriptional regulation could simply reflect the fact that the pool of CA involved in regulation is too small to detect in relation to the total cellular pool. The level of CA formed from elicitor-induced PAL activity depends on the relative activities of PAL and the enzymes removing CA (CA4H and conjugation enzymes) during the early stages of elicitation (Durst, 1976). PAL activity will obviously be low, and most of the initially formed CA may be channeled into other metabolites. CA4H is located in the microsomes, and the size of this microsomally channeled pool may be sensed in some manner and the signal transduced to regulate PAL gene expression. An alternative model could involve the

presence of a threshold concentration of CA or a metabolite/conjugate that is inhibitory to PAL gene activation. Elicitation would require a decrease in the concentration of this inhibitor along with a positive regulatory signal for the elicitation response. The kinetics of change in the levels of such a regulatory molecule would be rapid and transient, decreasing at the onset of PAL gene activation and returning above an inhibitory threshold level at the onset of the decline in the rate of PAL transcript appearance. Work is in progress to determine whether the levels of any CA derivatives might change in elicited cells in such a manner.

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LITERATURE CITED

Amrhein N, Gödeke K-H (1977) α-Aminooxy-β-phenylpropionic acid: a potent inhibitor of L-phenylalanine ammonia-lyase *in vitro* and *in vivo*. Plant Sci Lett **8:** 313–317

Bolwell GP, Bell JN, Cramer CL, Schuch W, Lamb CJ, Dixon RA (1985a) L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*. Characterization and differential induction of multiple forms from elicitor-treated cell suspension cultures. Eur J Biochem **149:** 411–419

Bolwell GP, Cramer CL, Lamb CJ, Schuch W, Dixon RA (1986) L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*: modulation of the levels of active enzyme by *trans*-cinnamic acid. Planta **169:** 97–107

Bolwell GP, Mavandad M, Millar DJ, Edwards KJ, Schuch W, Dixon RA (1988) Inhibition of mRNA levels and activities by *trans*-cinnamic acid in elicitor-induced bean cells. Phytochemistry **27:** 2109–2117

Bolwell GP, Robbins MP, Dixon RA (1985b) Metabolic changes in elicitor-treated bean cells. Enzymic responses associated with rapid changes in cell wall components. Eur J Biochem **148:** 571–578

Chomczynski P, Sacci N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162:** 156–159

Dalkin K, Edwards R, Edington B, Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.). I. Induction of phenylpropanoid biosynthesis and hydrolytic enzymes in elicitor-treated cell suspension cultures. Plant Physiol **92:** 440–446

Dixon RA, Browne T, Ward M (1980) Modulation of L-phenylalanine ammonia-lyase by pathway intermediates in cell suspension cultures of dwarf French bean (*Phaseolus vulgaris* L.). Planta **150:** 279–285

Dixon RA, Harrison MJ (1991) Activation, structure, and organization of genes involved in microbial defense in plants. Adv Genet **28:** 166–234

Dixon RA, Lamb CJ (1979) Stimulation of de novo synthesis of L-phenylalanine ammonia-lyase in relation to phytoalexin accumulation in *Colletotrichum lindemuthianum* elicitor-treated cell suspension cultures of French bean (*Phaseolus vulgaris*). Biochim Biophys Acta **586:** 453–463

Durst F (1976) The correlation of phenylalanine ammonia-lyase and cinnamic acid-hydroxylase activity changes in Jerusalem artichoke tuber tissues. Planta **132:** 221–227

Edwards R, Mavandad M, Dixon RA (1990) Metabolic fate of cinnamic acid in elicitor treated cell suspension cultures of *Phaseolus vulgaris*. Phytochemistry **29:** 1867–1873

Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, Dixon RA, Lamb CJ (1990) Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc Natl Acad Sci USA **87:** 9057–9061

Engelsma G (1974) On the mechanism of the changes in phenylalanine ammonia-lyase activity induced by ultraviolet and blue light in gherkin hypocotyls. Plant Physiol **54:** 702–705

Friend J (1979) Phenolic substances and plant disease. Rec Adv Phytochem **12:** 557–558

Gowri G, Paiva NL, Dixon RA (1991) Stress responses in alfalfa (*Medicago sativa* L.). 12. Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell cultures and developing plants. Plant Mol Biol **17:** 415–429

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol **40:** 347–369

Jorrin J, Lopez-Valbuena R, Tena M (1990) Effects of actinomycin D, cordycepin and cycloheximide on phenylalanine ammonia-lyase turnover in sunflower hypocotyls. J Plant Physiol **137:** 252–255

Kessmann H, Edwards R, Geno P, Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.). V. Constitutive and elicitor-induced accumulation of isoflavonoid conjugates in cell suspension cultures. Plant Physiol **94:** 227–232

Koster JA, Zuzok A, Barz W (1983) High performance liquid chromatography of isoflavones and phytoalexins from *Cicer arietinum*. J Chromatogr **270:** 392–395

Koukol J, Conn EE (1961) The metabolism of aromatic compounds in higher plants. IV. Purification and properties of the phenylalanine deaminase of *Hordeum vulgare*. J Biol Chem **236:** 2692–2698

Kroczek RA, Siebert E (1990) Optimization of northern analysis by vacuum-blotting, RNA-transfer visualization, and ultraviolet fixation. Anal Biochem **184:** 90–95

Lamb CJ (1979) Regulation of enzyme levels in phenylpropanoid biosynthesis: characterization of the modulation by light and pathway intermediates. Arch Biochem Biophys **192:** 311–317

Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. Mol Cell Biol **7:** 335–341

Loake GJ, Choudhary AD, Harrison MJ, Mavandad M, Lamb CJ, Dixon RA (1991) Phenylpropanoid pathway intermediates regulate transient expression of a chalcone synthase gene promoter. Plant Cell **3:** 829–840

Maniatis T, Fritsch EF, Sambrook J (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Mavandad M (1990) Regulation of the phenylpropanoid pathway by *trans*-cinnamic acid. PhD thesis, University of London, London, England

Mavandad M, Edwards R, Liang X, Lamb CJ, Dixon RA (1990) Effects of *trans*-cinnamic acid on expression of the bean phenylalanine ammonia-lyase gene family. Plant Physiol **94:** 671–680

Orr JD, Sumner LW, Edwards R, Dixon RA (1993) Determination of cinnamic acid and 4-coumaric acid in alfalfa (*Medicago sativa* L.) cell suspension cultures by gas chromatography. Phytochem Anal (in press)

Paiva NL, Edwards R, Sun Y, Hrazdina G, Dixon RA (1991) Stress responses in alfalfa (*Medicago sativa* L.). 11. Molecular cloning and expression of alfalfa isoflavone reductase, a key enzyme of isoflavonoid phytoalexin biosynthesis. Plant Mol Biol **17:** 653–667

Rademacher W, Fritsch H, Graebe JE, Sauter H, Jung J (1987) Tetcyclacis and triazole-type plant growth retardants: their influence on the biosynthesis of gibberellin and other metabolic processes. Pestic Sci **21:** 241–252

Schumacher HM, Gundlach H, Fiedlar F, Zenk MH (1987) Elicitation of benzophenanthridine alkaloid synthesis in *Escholtzia* cell cultures. Plant Cell Rep **6:** 410–413