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INHIBITION OF mRNA LEVELS AND ACTIVITIES BY *TRANS*-CINNAMIC ACID IN ELICITOR-INDUCED BEAN CELLS

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; elicitor; gene expression; phenylalanine ammonia-lyase; phenylpropanoid synthesis; transcription.

Abstract—Addition of *trans*-cinnamic acid to bean cell suspension cultures after their treatment with fungal elicitor resulted in the loss of induced phenylalanine ammonia-lyase (PAL) enzyme activity. In contrast elicitor-induced chalcone synthase (CHS) activity was arrested but did not decline, whereas chalcone isomerase (CHI) activity was relatively unaffected. However, translational activities of extracted polysomal mRNAs encoding these three enzymes were depressed by cinnamate treatments as were levels of PAL and CHS polysomal mRNAs and rates of transcription of these genes measured in isolated nuclei. Treatment of elicitor-induced cultures with L-α-aminooxy-phenylpropionic acid (AOPP), a potent inhibitor of PAL activity (and therefore cinnamate production) *in vivo*, resulted in increased production of PAL and CHS mRNAs. Addition of cinnamate to elicitor-treated cultures inhibited the appearance of a number of polypeptides translated *in vitro* from polysomal mRNA, although at least nine polypeptides were specifically induced by cinnamate treatment. We conclude that cinnamic acid potentially could act as an *in vivo* modulator of the synthesis of phenylpropanoid pathway enzymes although it is not yet fully possible to rule out less specific inhibitory effects.

INTRODUCTION

L-Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyses the first reaction in the biosynthesis of plant phenolic compounds from L-phenylalanine [1]. Treatment of suspension cultured bean cells wtih elicitor macromolecules heat-released from cell walls of the fungal phytopathogen *Colletotrichum lindemuthianium* leads to rapid induction of enzyme activities involved in the accumulation of isoflavonoid phytoalexins derived from L-phenylalanine [2–6]. This response is characterized by rapid increases in PAL and chalcone synthase (CHS) mRNA levels and translational activities leading to increased rates of enzyme synthesis [2, 4, 7, 8], and the differential induction of multiple forms of active PAL differing in pI and K_m values [9]. The elicitor-induced increase in PAL activity is rapidly reversed by addition of *trans*-cinnamic acid, the product of the PAL reaction [10].

Density labelling studies with ^2H from ^2H_2O have indicated that the loss of PAL activity observed on application of exogenous cinnamate to pea epicotyl sections is mediated by a dual mechanism involving a decrease in the rate of PAL synthesis and an increase in the rate of PAL removal [11]. Confirmation of the cinnamate-mediated inhibition of PAL synthesis has been obtained from studies on the incorporation of ^{35}S-methionine into immunoprecipitable PAL subunits *in vivo* in elicitor-treated bean cultures [12]. Furthermore, a mechanism has recently been proposed for the cinnamate-mediated removal of PAL activity involving an irreversible inactivation of the enzyme in the absence of an increased rate of subunit degradation [12].

A number of studies have suggested that endogenous cinnamate pools may act *in vivo* to modulate PAL turnover. Central to this argument is the observed superinduction of PAL enzyme activity by treatments, such as application of the potent PAL inhibitor AOPP, which prevent the accumulation of endogenous cinnamate *in vivo* [10, 13–16]. That cinnamate potentially may act to regulate the flux through the phenylpropanoid pathway at sites other than PAL is suggested by the induction of the activities of chalcone isomerase (CHI) [16] and hydroxycinnamoyl CoA: quinate hydroxycinnamoyl transferase [17], in cinnamate-treated tissues of bean and potato respectively.

In the present paper we extend previous observations on the inhibition of PAL synthesis by exogenously applied cinnamate to consider transcriptional events. We also describe the effects of cinnamate on mRNAs encoding other phenylpropanoid pathway enzymes. The results are discussed in relation to the selectivity and possible involvement *in vivo* of cinnamate as a modulator of plant gene expression.

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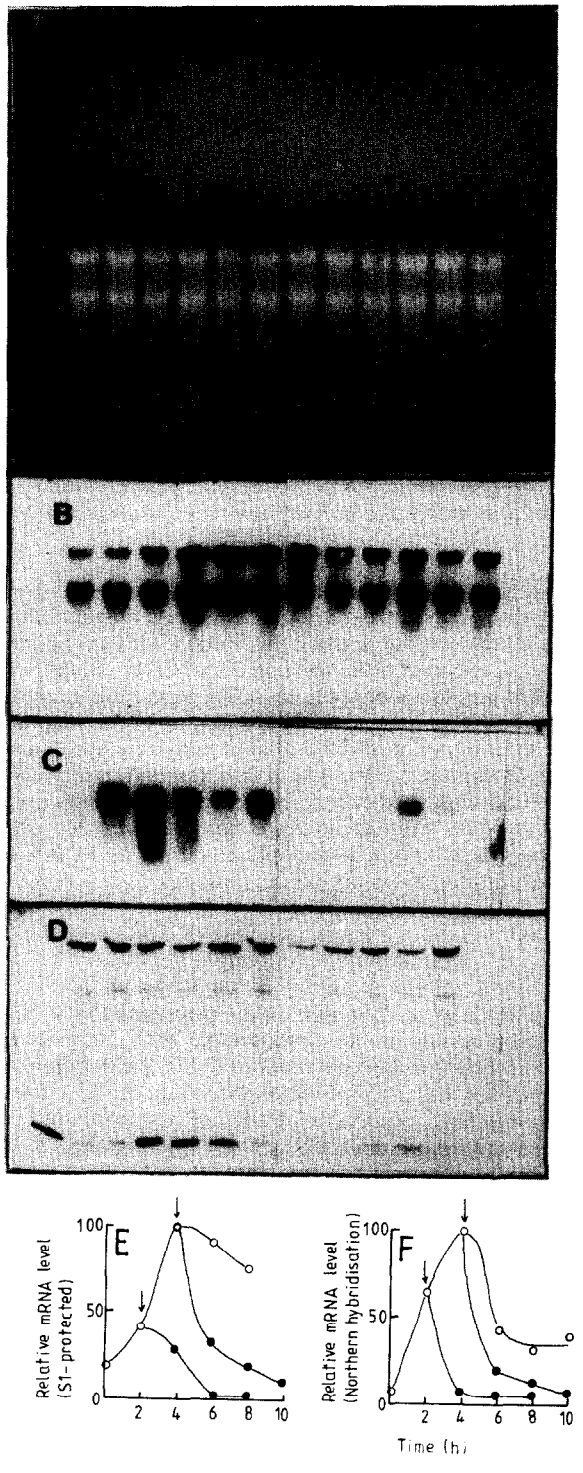

Fig. 1. Levels of PAL and CHS transcripts in elicitor-treated bean cell suspension cultures without or wtih addition of cinnamic acid (1 mM, added at the times shown by the arrows in E and F). A–C Northern blot analysis of CHS mRNA. Polysomal RNA was prepared from cells 0 hr (Track 1) 2 hr (2), 4 hr (3), 6 hr (4), 8 hr (5) and 10 hr (6) after addition of elicitor alone, 4 hr (Track 7), 6 hr (8) and 8 hr (9) after addition of cinnamate at 2 hr, and 6 hr (Track 10), 8 hr (11) and 10 hr (12) after addition of cinnamate at 4 hr. RNA was separated on a 1% agarose gel and stained with acridine orange (A). It was then transferred to a PALL Biodyne membrane and hybridized with labelled insert from a ribosomal RNA cDNA clone (B) or from p CHS5. (C). D shows S1 nuclease protection analysis of steady-state PAL transcript levels. The probe used was constructed from the sequence spanning the intron-exon boundary of the PAL genomic clone gPAL2. Similar results were obtained with other PAL constructs described in Experimental. The arrow indicates the PAL-specific protected fragment. Tracks are as in A above. E and F. PAL (E) and CHS (F) mRNA levels calculated from densitometric scanning of autoradiographs as in C and D above.

RESULTS

General effects of addition of exogenous cinnamic acid

Addition of cinnamic acid (1 mM) to cell cultures had no significant inhibitory effect on total incorporation of [^{35}S] methionine into protein *in vivo* [12]. There was also no inhibitory effect on total polysomal RNA levels, on the intactness of rRNA analysed on 1% agarose gels (see Fig. 1, A and B), or on the level of incorporation or length of transcript (80–2000 bp) in nuclear transcript run off experiments (data not shown). However, the mRNA population appeared to change qualitatively in response to cinnamate treatments, as reflected by the patterns of polypeptides synthesised *in vitro* from polyA$^+$ or total polysomal RNA (Figs 2 and 3). The overall patterns of translation products from polyA$^+$ RNA differed depending upon whether cinnamic acid was added at 2 or 4 hr after elicitor (Fig. 2). Although 1D gels do not fully resolve the qualitative changes, it can be seen that cinnamic acid addition at 2 hr predominantly prevented increases in elicitor-induced polypeptides (particularly CHS subunits at M_r 43 000), whereas this was not the case when cinnamate was added 4 hr post elicitation. In addition, cinnamate appeared to induce the appearance of some polypeptides. This was investigated in more detail by 2D IEF: SDS PAGE analysis of *in vitro* translation products from polysomal mRNA isolated from untreated and cinnamate treated cells (Fig. 3). At least nine polypeptides were resolved which appeared to be induced in response to cinnamate, ranging in M_r from 16 000 to *ca* 100 000. These data indicate that cinnamate is not a blanket inhibitor of new mRNA production. The lack of any severe general metabolic effects as a result of cinnamate addition was also reflected in the similarity of total translational activities of mRNA from cinnamate-treated, and untreated, elicited cells (data not shown).

Effects of cinnamate on the extractable activities of phytoalexin biosynthetic enzymes

Addition of cinnamic acid to cultured bean cells brought about rapid loss of extractable PAL activity (Fig. 4A), and this results in part from inhibition of PAL subunit synthesis *in vivo* [12]. In contrast, the elicitor-induced increase in the extractable activity of CHS is halted but not reversed by cinnamic acid (Fig. 4B), and in the case of CHI, enzyme activity is unaffected by cinnamate treatments 4 hr after addition of elicitor (Fig. 4C). Indeed, under conditions of low endogenous cinnamate pool size, CHI activity may be induced by exogenous cinnamate additions [16]. The activity profiles for both CHS and CHI suggest that the effects observed after

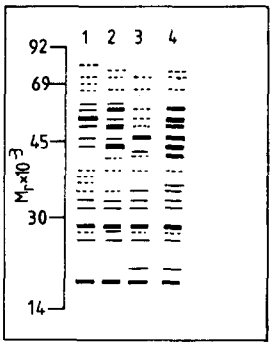

Fig. 2. Schematic representation of fluorographs showing polypeptides synthesized *in vitro* from polyA$^+$ mRNA extracted from elicitor-treated cells sampled at (1) 0 hr, (2) 4 hr, (3) 4 hr after cinnamate addition at 2 hr and (4) 6 hr after cinnamate addition at 4 hr. The extent of shading of the bands is an approximation of their relative intensities. Bands present in minus RNA controls are not shown.

addition of cinnamate at 2 hr could be accounted for by a delay followed by increased synthesis, whereas the effect on PAL activity clearly involves an extra component of inactivation, as has been previously demonstrated [12]. The induced synthesis of all three enzymes by elicitor has been previously shown to result from increased gene activation processes [3–5, 8] although the induction of CHI activity may also involve activation of preformed inactive enzyme [18, 19]. The underlying basis for the rapid loss, or cessation of appearance, of the increased enzyme activities in response to elicitor action brought about by addition of cinnamic acid has now therefore been investigated by measurement of the corresponding transcript levels and translatable mRNA activities.

Changes in mRNA species as a consequence of the addition of cinnamic acid

Changes in mRNA activity were measured by immunoprecipitation of [^{35}S] methionine-labelled enzyme subunits synthesized *in vitro* by translation of polysomal RNA prepared at various times following exposure of cells to elicitor with or without addition of cinnamic acid (Fig. 4D–F). In untreated cells, only low levels of activity of the mRNAs encoding the enzymes of phytoalexin biosynthesis could be detected. Addition of elicitor brought about transient increases in the activities of these mRNAs with maximum activities 2–6 hr after treatment, followed by subsequent decay. Additions of exogenous cinnamic acid resulted in rapid losses in the translatable activities of mRNAs encoding PAL, CHS and CHI, if added 2 hr after exposure to elicitor. Addition at 4 hr resulted in rapid loss of CHI translatable activity, but far lesser effects on PAL and CHS.

Levels of PAL and CHS mRNAs were also measured by Northern hybridization analysis, and, in the case of PAL (which yields a weak signal on Northern blots) by S1 nuclease protection assays. In these experiments, the elicitor-induced mRNAs reached maximum levels at a later time point (4 hr instead of 2 hr). The results (Fig. 1) clearly confirm that exogenously applied cinnamic acid brings about rapid loss in the levels of the mRNAs encoding PAL and CHS.

Effects of inhibition of endogenous cinnamate production on the levels and translational activities of mRNA species

L-α-Aminooxy-β-phenylpropionic acid (AOPP) is a powerful competitive inhibitor of PAL whose application to bean cells *in vivo* results in superinduction of PAL activity [16]. The effect of addition of AOPP to bean cells 2 or 4 hr after elicitation is shown in Fig. 5. In direct contrast to the effect of application of cinnamic acid, the extractable PAL activity increased above the elicitor-induced level after a lag period of *ca* 2 hr (even though the enzyme may be fully inhibited *in vivo*). The underlying basis for this effect probably resides in increased synthesis of PAL subunits, as suggested by the increased levels of PAL mRNA measured by immunoprecipitation of subunits synthesized *in vitro* or by S1 nuclease protection assays (Fig. 5B–D). Furthermore, AOPP treatment also brought about an increased induction of CHS mRNA level as measured by Northern hybridization, at the same time points at which superinduction of PAL mRNA was observed (Fig. 5C, square and triangular symbols). AOPP treatment had no obvious qualitative effect on the patterns of polypeptides induced by elicitation, as far as could be assessed by 1D gel analysis (data not shown).

Effect of cinnamate on transcription in isolated nuclei

To assess whether the action of cinnamic acid involves effects on the transcription rates of PAL and CHS genes or on early post-transcriptional events, nuclear run-off transcript experiments were performed with nuclei isolated from bean cells three hours after elicitor treatment, with or without addition or cinnamate after one hour, and with nuclei from unelicited controls. The cell batch used was the one which exhibited maximum PAL and CHS transcript levels at 4 hr post elicitation (Fig. 1). Specific [^{32}P]-labelled transcripts were isolated by hybridization to immobilised cDNA insert sequences under conditions (1 μg DNA) of excess plasmid insert. Under these conditions, non-specific binding of any RNAs to unrelated vector sequences, e.g. pBR322 similarly immobilized, was negligible and only hybridization of specific PAL or CHS transcripts from nuclei from elicited cells could be visualized by autoradiography.

Relative amounts of [^{32}P]-labelled RNA hybridized to cDNA inserts corresponding to PAL or CHS mRNA were measured by scintillation counting. After subtraction of background and correction for cpm added and radioisotope decay, nuclei from elicited cells showed about a six to ten-fold increase in transcriptional activity compared with those from unelicited cells (Table 2). A 2 hr exposure of the cells to cinnamate reduced the transcription rates of PAL and CHS mRNAs, the effect being most noticeable for CHS. This may reflect differences in the time of attainment of maximum rates of transcription for these two gene families in this experiment.

Effects of addition of cinnamate or AOPP on phenolic metabolism in elicited plant cells

Effects of cinnamic acid or AOPP addition on general phenolic metabolism in bean cells are shown in Table 2. Both treatments radically lowered or abolished phytoalexin production. Addition of cinnamic acid appeared to lead to preferential metabolism into wall phenolics in contrast to the result of AOPP treatment, which led to a

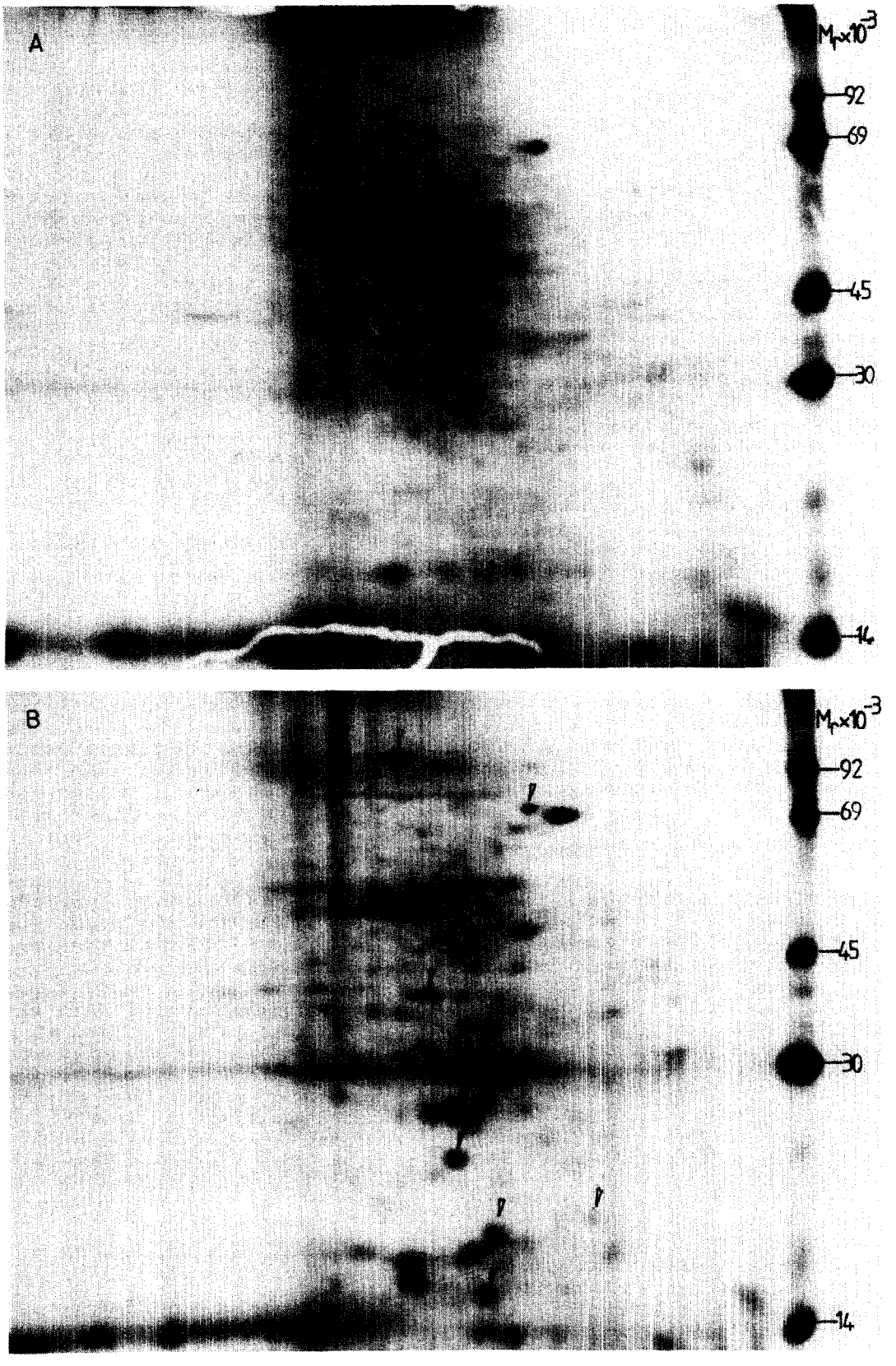

Fig. 3. 2D IEF:SDS PAGE analysis of ^{35}S-labelled polypeptides synthesized *in vitro* from polysomal mRNA isolated from cultured bean cells in the absence of cinnamic acid (A) or in the presence of 1 mM cinnamic acid for 6 hr (B). The polarity shows basic polypeptides on the right. Cinnamic-acid-induced polypeptides in (B) are marked with arrowheads.

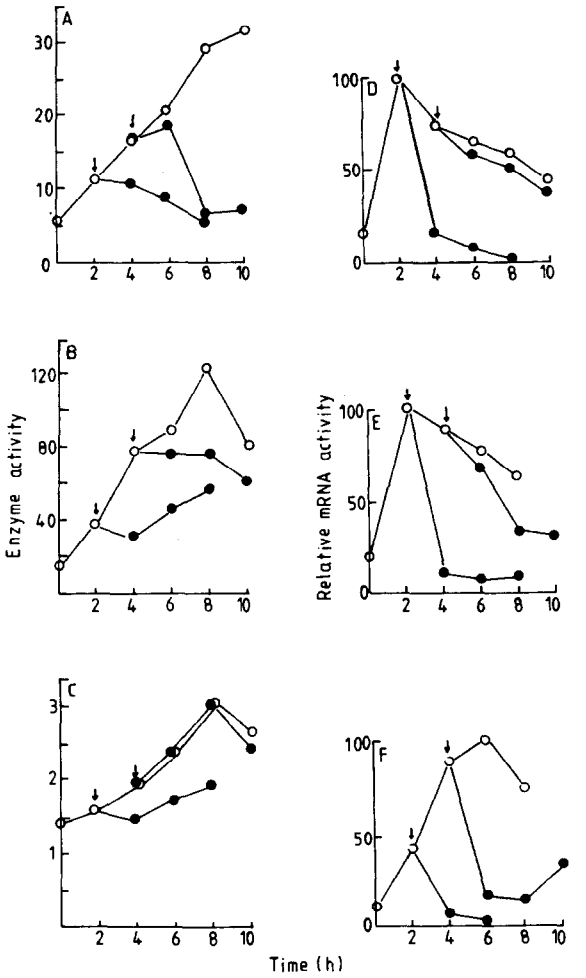

Fig. 4. The effects of cinnamic acid (1 mM, added at the times shown by arrows) on the extractable enzyme activities (A–C) and relative mRNA activities (D–F, determined by *in vitro* translation of polysomal mRNAs followed by immunoprecipitation with monospecific antisera) of PAL (A,D), CHS (B, E) and CHI (C,F) in elicitor-treated bean cell suspension cultures. Enzyme activity units are μkat/kg protein (PAL, CHS) or mkat/kg protein (CHI).

lowered appearance of these products. Thus, even though AOPP may cause increased appearance of transcripts coding for CHS involved in the phytoalexin pathways, the block on the flux through the phenylpropanoid pathway at the level of PAL may be sufficient to prevent the appearance of phytoalexin and wall phenolics. Cinnamate additions bring about the down-regulation of the appearance of transcripts coding for PAL and the enzymes CHS and CHI specific to the phytoalexin pathway, inhibiting production of phytoalexins. In contrast, enzyme activities leading to wall phenolic production must be relatively unaffected. Differential effects of cinnamate on individual sites in overall phenolic metabolism are therefore indicated. Further analysis will be necessary in order to establish whether quantitative changes in wall-bound phenolics also reflect qualitative differences.

DISCUSSION

Although much attention is currently being paid to the molecular mechanisms leading to increased gene expression in plant cells, relatively little has been directed towards the processes which underlie the cessation of gene transcription and/or enzyme synthesis and the possible increased rate of removal of enzyme activity. In addition to constituting an important component of the overall process of gene regulation in plants, knowledge of the factors which signal down-regulation of plant genes and enzyme removal may be of great value in devising strategies for the maximisation of the biosynthetic potential of plant cells. A number of studies have provided evidence which suggests that intracellular levels of *trans*-cinnamic acid, or some metabolite of it, may act as a signal for the regulation of the flux through the phenylpropanoid pathway. The effects of exogenous additions of cinnamic acid, which suppress the extractable activity of PAL *in vivo* [10, 12] and L-α-aminooxy-β-phenylpropionic acid, the powerful competitive inhibitor of PAL, whose application can result in superinduction of extractable PAL activity [13], could be interpreted in terms of the putative involvement of cinnamic acid in a dual control mechanism involving inhibition of the rate of PAL synthesis and stimulation of the rate of PAL removal. Both processes were initially revealed by density labelling studies with ^2H from ^2H_2O [11, 20].

In the present work, Northern hybridization, S1 nuclease-protection and nuclear transcript run-off analyses have demonstrated an inhibition of transcription of PAL and CHS mRNAs and/or rapid removal of hybridizable mRNA following addition of cinnamic acid to elicitor-induced cells. The extent of the loss in mRNA activity or steady state level appears to depend on the time of addition of cinnamic acid in relation to the time of attainment of maximum mRNA activity/level (compare Fig. 4 D–F and 1E and F),the greater loss at early time points supporting an effect on mRNA appearance rather than half-life, although it should be noted that maximum rates of PAL and CHS transcription would clearly have been attained by 2 hr in Fig. 4 and by 4 hr in Fig. 1 (although elevated rates of transcription for both PAL and CHS may still be measured 4 hr after elicitation [21]). As a consequence of these effects the translatable mRNA levels encoding PAL, CHS and CHI decline resulting in decreased synthesis of immunodetectable enzyme subunits *in vitro*. These changes occur against a background of no gross quantitative effects on mRNA and protein synthesis, although the overall patterns of polypeptides newly synthesised differ qualitatively in the presence and absence of cinnamate, and a number of specific cinnamate-induced polypeptides are clearly resolved by 2D gel analysis of *in vitro* translation products (Fig. 3). Previous authors [22] have argued that cinnamate acts as a general inhibitor of translational activity, while at the same time stimulating PAL mRNA levels, a finding in direct contradiction to the results presented here. These effects of cinnamate were observed after much longer time periods than those investigated here, and involved use of a PAL cDNA probe which revealed mRNA induction kinetics at variance with kinetics of appearance of translatable mRNA activity [23].

Although cinnamate is clearly not a blanket, non-selective transcriptional inhibitor in our experiments, results to date do not, however, rule out general effects resulting from, for example, cinnamate toxicity. It could

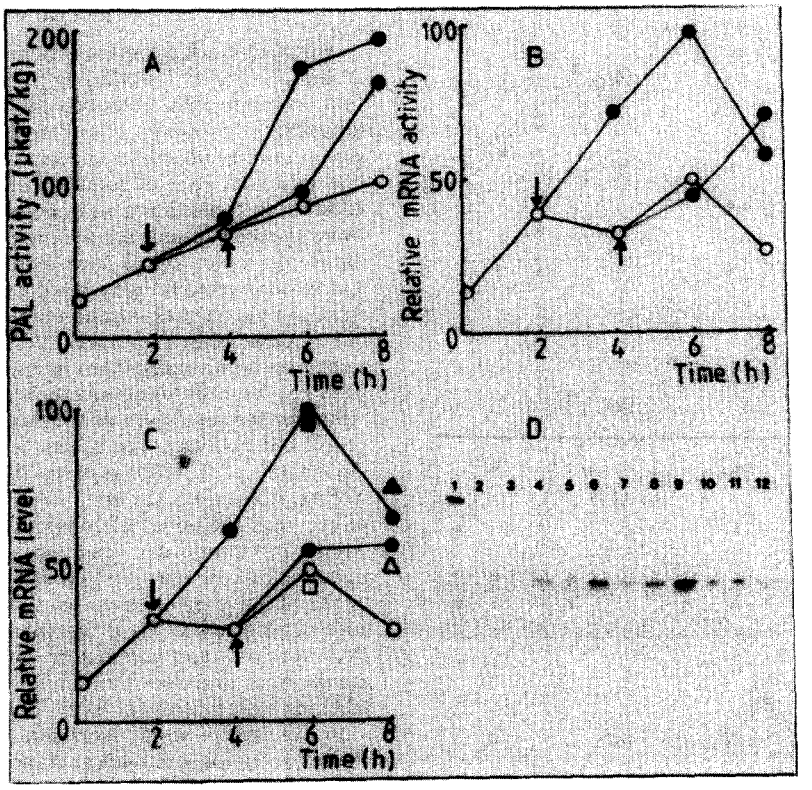

Fig. 5. The effect of AOPP (1 μM) on elicitor induction of PAL and CHS. ○——○ = cells treated with elicitor alone (30 μg glucose equivalents/ml). ●——● = cells treated with elicitor followed by addition of AOPP as indicated by the arrows. A: PAL enzyme activity; B: translatable PAL mRNA activity as determined by *in vitro* translation analysis; C: level of PAL transcripts as determined by S1 nuclease protection assay (see D). The level of CHS transcripts measured by Northern hybridization are also shown for untreated (open symbols) and AOPP treated (closed symbols) cells. (—■—) AOPP added at 2 hr and cells sampled at 6 hr (—▲—) AOPP added at 4 hr and cells sampled at 8 hr (□, △ = non-AOPP-treated controls). D: S1 nuclease protection assay for PAL mRNA. The probe was constructed from the 3′ untranslated region of pPAL5. Track (1) = undigested probe (2) = no RNA. In tracks 3–12, elicited cells were analysed at (3) 0 hr (4) 2 hr (5) 4 hr (6) 6 hr and (7) 8 hr without AOPP, with AOPP added at 2 hr and sampled at (8) 4 hr (9) 6 hr and (10) 8 hr, and with AOPP added at 4 hr and sampled at (11) 6 hr and (12) 8 hr.

Table 1. Transcription *in vitro* by nuclei isolated from cultured bean cells subjected to elicitor and cinnamate treatments

	dpm hybridized/10^6 dpm transcripts		
Cell treatment Probe	No elicitor	Elicitor	Elicitor + cinnamate (1 mM)
pPAL5	98 ± 13	678 ± 65	440 ± 175
pCHS5	296 ± 37	2729 ± 776	725 ± 512

Cells were exposed to cinnamate (where it was used) 1 hr after elicitation, and all cells were harvested 3 hr after elicitation. Nuclei were isolated, allowed to transcribe for 30 min, and transcripts analysed as described in Experimental.

always be argued that some of the polypeptides newly synthesized in response to exogenous cinnamate treatments are 'stress proteins' or enzymes of some detoxification mechanism. Indeed, previous results have indicated rapid esterification of exogenously added free cinnamate in bean cells [34]. The toxicity argument could, however, be used in support of a physiological role for endogenous cinnamate as an inhibitor of its own synthesis *in vivo*. The weaker effects of cinnamate addition 2 hr post elicitation, and the total lack of inhibitory effect at 4 hr, on the translational activity of a mRNA species encoding a putative cytochrome P450 (data not shown) are suggestive of some degree of specificity for inhibition of appearance of elicitor-induced polypeptides by cinnamate.

Inhibition of cinnamate formation *in vivo* by AOPP

Table 2. Effects of cinnamic acid and AOPP on phenolic metabolism in bean cells

Treatment*			Maximum enzyme activities			Level of phenolic products after 24 hr	
Elicitor	Cinnamic acid	AOPP	PAL (μkat/kg protein)	CHS (μkat/kg protein)	CHI† (mkat/kg protein)	Wall-bound phenolics (A310/g fr. wt)	Phaseollin (μg/g fr. wt)
—	—	—	16	21	2.5	7	0
+	—	—	55	121	6	20	277
—	+	—	3	—	—	34	0
+	+	—	4	58	8	34	0
—	—	+	28(0)	—	—	6	0
+	—	+	109(0)	—	5.5	16	23

*Elicitor was added at zero time and cinnamate (1 mM) and AOPP (1 μM) additions made at 2 hr. Enzymes were assayed at subsequent time points (8 hr, PAL and CHS, 24 hr CHI) and the maximum recorded activities are shown. Figures in parentheses for PAL show the putative *in vivo* activity.

†From ref. [16].

resulting in increases in PAL and CHS mRNAs is fully consistent with a model in which intracellular cinnamate levels act as a signal for the flux through the phenylpropanoid pathway at the level of gene expression. These experiments do not involve exogenously applied cinnamate, and AOPP, which is a largely specific PAL inhibitor, is applied at very low concentrations (1 μM). Furthermore, the demonstration of cinnamate-mediated irreversible inactivation of PAL [12], brought about by a proteinaceous factor whose synthesis is induced by cinnamate (Mavandad, M. and Dixon, R. A., unpublished results), suggests that cinnamate may exert specific positive as well as negative effects relating directly to expression of the phenylpropanoid pathway; enzyme inactivation in response to exogenous cinnamate additions is, as far as can be assessed at present, specific for PAL. The behaviour of CHS and CHI activities reflects inhibition of synthesis rather than effects on removal of activity, although the total lack of effect of cinnamate on CHI extractable activity when added 4 hr post elicitation may possibly reflect activation of pre-existing enzyme, a process previously demonstrated for this enzyme [18, 19]. Under certain conditions, cinnamate may act as an inducer of CHI extractable activity in cultured bean cells [16], and light-induced increases in the extractable activity of hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase have been linked to increases of the cinnamate pool in potato [17], further supporting a regulatory role for this molecule *in vivo*. Furthermore, previous work [12] had indicated that endogenous levels of cinnamate can reach those observed with exogenous additions, and that an approximately reciprocal relationship exists between free cinnamate levels and extractable PAL activity in bean cells.

Taking all the evidence together, it is possible that the down-regulatory effects on mRNA levels/activities observed in the present work may well occur *in vivo* under circumstances which lead to elevated levels of cinnamic acid, or some metabolite or bound form of it, mediated by the transient induction of PAL. These findings at least suggest mechanisms by which cinnamate may act to control the flow of phenylalanine into phenylpropanoid secondary products. In order to assess the validity of this model it will be necessary to examine cinnamate pools, ratios of active to inactive enzymes (e.g. PAL and CHI) and appearance of cinnamate-inducible polypeptides/mRNA species in bean cells during transient induction of the phenylpropanoid pathway in the absence of exogenously added cinnamate, and to obtain further information on effects of lower concentrations of cinnamate (which still block PAL and CHS transcripts, unpublished data) on elicitor-induced and uninduced genes. These studies are now in progress.

EXPERIMENTAL

Materials, antisera and DNA probes. [α-^{32}P]-dCTP (400 Ci/mmol) and [α-^{32}P]-UTP (400 Ci/mmol) were obtained from Amersham International U.K. Monospecific antisera were to PAL [9] and CHI [19] from elicitor-treated bean cell cultures and to chalcone synthase from light-induced cell cultures of *Petroselinum hortense* [24]. cDNAs complementary to PAL (pPAL5) and CHS (pCHS5) sequences were as described [8, 24]. These clones do not distinguish between different members of the bean PAL and CHS multigene families. cDNA (pTA71) to rRNA was obtained from a cDNA library of sequences complementary to wheat RNA; this cross-hybridises with bean rRNA.

Growth and elicitation of plant cell cultures. Cell suspension cultures of bean (*Phaseolus vulgaris* cv. Canadian wonder) were grown as described [25] except that cultures were maintained in total darkness. All experiments were with cultures in exponential growth phase. Cells were exposed to a crude elicitor preparation, heat released from the cell walls of *Colletotrichum lindemuthianum* as described [26], at a final concentration of 30 μg glucose equivalents/ml culture. Cinnamate treatments were carried out as described [12]. Cells were harvested by vacuum filtration, frozen in liquid N_2 and stored at −70° until required.

Enzyme extraction and assay. Extraction and assay of PAL and CHS [2] and chalcone isomerase [27] were as described. One unit of enzyme activity (1 kat) is defined as the amount of enzyme required for the formation of 1 mol of product in 1 sec under the assay conditions.

Isolation of RNA, and protein synthesis in vitro. Polysomal RNA for *in vitro* translation was isolated by a modification [24]

of the method of [28]. Concns of RNA were determined spectrophotometrically at 260 nm.

Isolated polysomal RNA was translated *in vitro* in presence of [^{35}S] methionine using an mRNA dependent rabbit reticulocyte lysate translation system (Amersham International U.K.), and incorporation of [^{35}S] methionine into total protein measured as described [7]. Enzyme subunits were separated from other translation products by indirect immunoprecipitation with appropriate antisera and protein A Sepharose followed by SDS-polyacrylamide gel electrophoresis [7, 9, 19]. Enzyme subunits were located by fluorography and [^{35}S] methionine incorporation determined as previously described [2]. 2D IEF:SDS PAGE analysis was performed as described [9]. mRNA activity is defined as the incorporation of [^{35}S]-methionine into immunoprecipitable enzyme subunits as a percentage of incorporation into total protein. Temporal changes in mRNA activity are expressed relative to mRNA activity at time of maximal induction [4].

Analysis of RNA. RNA was denatured with glyoxal, separated by electrophoresis on 1.0% agarose gels in 10 mM KH_2PO_4 pH 7.0 [29] and blotted onto PALL Biodyne nylon membranes (PALL Portsmouth U.K.). Blots were hybridized with probes prepared by nick translation [30] of the 1500 bp pPAL5 cDNA, the 1200 bp pCHS5 cDNA or the 1400 bp wheat ribosomal RNA cDNA. Hybridization was performed at 40° in a buffer containing 50% formamide, 0.75M NaCl, 0.75M Na citrate (pH 7.0), 0.1% polyvinyl pyrollidone, 0.1% bovine serum albumin, 5mM EDTA, 0.1% $NaDodSO_4$ and 100 μg herring sperm DNA per ml. Filters were pre-incubated for 24 hr in buffer without probe followed by hybridization for 48 hr in the presence of ^{32}P-labelled probe. Following autoradiography, mRNA was quantified by scanning densitometry.

SI nuclease protection assays were performed by the method of [31]. A general PAL (non-gene specific) probe constructed from the Hind III-Hinc II restriction fragment spanning the intron-exon boundary of PAL genomic clone gPAL2, or specific probes incorporating (a) a fragment of gPAL2 specific for the 3′ untranslated region or (b) the untranslated region of pPAL5, were cloned into M13 mp8. After priming and elongation, probes were excised with Hinc II and separated on a 4% polyacrylamide gel. Bands were cut out, electroeluted, pptd with EtOH and re-dissolved in 200 μl 50% formamide, 0.4 M NaCl, 1 mM EDTA in 20 mM PIPES buffer pH 6.4 before hybridization at 50° overnight. Following Sl nuclease-treatment, protected bands were analysed on a 6% acrylamide gel in the presence of 7 M urea and detected by autoradiography.

Isolation of nuclei and transcript 'run offs'. Cultured bean cells were harvested and incubated for 30 min in 2.5 vol. of 0.7 M mannitol, 10 mM MES buffer pH 6.0 containing 5 mM EDTA, 0.1% (w/v) bovine serum albumin, 0.2 mM PMSF, 10 mM vanadyl ribonucleosides, 0.1% (w/v) cellulase and 0.5% (w/v) pectinase. They were then washed twice by resuspension and centrifugation at 300 *g* for 5 min in incubation buffer without enzymes. The final pellet of cells was resuspended in 2.5 vol. of 0.25 M sucrose, 10 mM NaCl, 10 mM MES pH 6.0, 5 mM EDTA, 0.25 mM spermine–HCl, 0.5 mM spermidine phosphate, 20 mM 2-mercaptoethanol, 0.2 mM PMSF, 0.6% Triton X-100, 10 mM vanadyl ribonucleosides and 50% glycerol and homogenized × 3 for 15 sec in an Ultraturrax homogeniser. The homogenate was filtered successively through 100 μm, 60 and 40 μm nylon mesh and then centrifuged at 4000 *g* for 10 min. The pellet, which contained starch grains and nuclei, was resuspended in 20 ml 95% Percoll (Sigma, U.K.) containing 25 mM MES pH 6.5, 0.25 M mannitol, 1 mM dithiothreitol, 10 mM KCl, 5 mM $MgCl_2$ and 0.6% (v/v) Nonidet P-40. The nuclei were harvested from the top of the Percoll gradient and resuspended in 20 ml 10 mM MES pH 6.5, 0.3 M sucrose, 5 mM $CaCl_2$, 5 mM 2-mercaptoethanol and 0.6% Nonidet P40 before centifugation at 4000 *g* for 10 min. After washing and centrifugation in the same buffer, they were resuspended in 50 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, 50% glycerol at a final concentration of 2×10^8 nuclei/ml.

Nuclei (10^7) were incubated with 50 mM Tris–HCl pH 7.8, 5 mM $MgCl_2$, 75 mM $(NH_4)_2SO_4$, 8% glycerol, 0.5 mM each of ATP, GTP, CTP and 50 μCi [α– ^{32}P] UTP (400 Ci/mmol) in a total volume of 100 μl at 26° for 30 min. Transcription was followed by removing 2 μl aliquots at intervals and spotting onto glass fibre paper discs (Whatman GF3) which were then washed in ice cold 10% (w/v) TCA, 1% (w/v) $Na_4P_2O_7$ for 10 min followed by two washes in ice-cold 5% (w/v) TCA, 1% (w/v) $Na_4P_2O_7$ and one final rinse in Me_2CO. Filters were dried and radioactivity measured by scintillation counting. Incorporations were linear up to 30 min.

Transcribed RNA was isolated by the method of ref. [32] and analysed on a 4% polyacrylamide gel in the presence of 7 M urea prior to autoradiography. Alternatively, *in vitro* labelled transcripts were passed through Sephadex G50 in 10 mM Tris–HCl pH 7.5 containing 0.3 M NaCl, 0.1% $NaDodSO_4$, 1 mM EDTA to remove residual unincorporated nucleotide. Samples were treated in hybridization buffer (50% formamide, 0.75 M NaCl, 0.075 M Na citrate (pH 7.0), 0.1% polyvinylpyrollidone, 0.1% bovine serum albumin, 5 mM EDTA, 0.1% $NaDodSO_4$, 100 μg/ml herring sperm DNA and 0.1 μg/ml polyadenylic acid) at 50° for 5 min before hybridization against pPAL5 and pCHS5 cDNA inserts that had been Southern transferred onto PALL biodyne membrane squares (1 μg insert per sample of RNA). Hybridization conditions and washings were as described in [23]. Binding was checked by autoradiography, and the amount of radioactivity bound determined by scintillation counting.

Other procedures. Protein was determined by the method of [33] and wall bound phenolics as described in [34]. Bean phytoalexins were extracted in BDH Spectrosol grade EtOH (10 ml/g fresh weight of cells). The homogenate was filtered through a 0.22 μm membrane filter and the filtrate evapd to dryness *in vacuo*. The residue was resuspended in EtOH and separated by HPLC on a Partisil 10 ODS 1 reverse phase column with a 20–80% MeCN in H_2O gradient at 1.6 ml/min over 30 min. Sample elution was monitored at 280 nm and peak areas compared with a standard phaseollin solution.

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