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Co-ordinated synthesis of phytoalexin biosynthetic enzymes in biologically-stressed cells of bean (*Phaseolus vulgaris* L.)

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Changes in the rates of synthesis of three enzymes of phenylpropanoid biosynthesis in *Phaseolus vulgaris* L. (dwarf French bean) have been investigated by immunoprecipitation of [^{35}S]methionine-labeled enzyme subunits with monospecific antisera. Elicitor causes marked, rapid but transient co-ordinated increases in the rate of synthesis of phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase concomitant with the phase of rapid increase in enzyme activity at the onset of accumulation of phenylpropanoid-derived phytoalexin antibiotics in suspension cultures of *P. vulgaris*. Co-ordinate induction of enzyme synthesis is also observed in hypocotyl tissue during race:cultivar-specific interactions with *Colletotrichum lindemuthianum*, causal agent of anthracnose. In an incompatible interaction (host resistant) there are early increases apparently localized to the initial site of infection prior to the onset of phytoalexin accumulation and expression of hypersensitive resistance. In contrast, in a compatible interaction (host susceptible) there is no induction of synthesis in the early stages of infection, but a delayed widespread response at the onset of lesion formation associated with attempted lesion limitation. It is concluded that expression of the phytoalexin defense response in biologically stressed cells of *P. vulgaris* characteristically involves co-ordinate induction of synthesis of phytoalexin biosynthetic enzymes.

Key words: chalcone isomerase/chalcone synthase/enzyme synthesis/phenylalanine ammonia-lyase/plant disease resistance

Introduction

Plant disease resistance involves not only static protection but also active defense mechanisms prominent amongst which is the induced accumulation of host-synthesised phytoalexin antibiotics (Bell, 1981; Dixon *et al.*, 1983a; Sequeira, 1983). Phytoalexin accumulation, which is largely a result of increased synthesis from remote precursors (Moesta and Grisebach, 1980, 1981), can be induced not only by infection but also by glycan, glycoprotein and lipid elicitors isolated from fungal and bacterial cell walls and culture filtrates and by a variety of structurally unrelated, artificial inducers (Dixon *et al.*, 1983a; West, 1981).

In cell suspension cultures of *Phaseolus vulgaris* L. (dwarf French bean) elicitor treatment causes marked, rapid increases in the activity levels of at least five enzymes of phenylpropanoid biosynthesis concomitant with the onset of accumulation of phaseollin and structurally-related isoflavonoid phytoalexins (Dixon and Bendall, 1978; Dixon and Lamb, 1979). *In vivo* labeling coupled with specific immunoprecipitation has shown that elicitor causes a marked but transient increase in the rate of synthesis of chalcone synthase (Lawton *et al.*, 1983a) which catalyses the first reaction of phenylpropanoid biosynthesis specific to a flavonoid/isoflavonoid branch pathway (Dixon *et al.*, 1983a; Whitehead *et al.*, 1982). Increases in the rate of enzyme synthesis can be observed within 20 min of elicitor treatment with maximum rates being obtained 3–4 h after elicitation at which time chalcone synthase accounts for ~1% of total protein synthesis. *In vitro* translation of isolated RNA indicates that elicitor induction of enzyme synthesis arises mainly if not exclusively from an increase in the activity level of the mRNA encoding chalcone synthase (Lawton *et al.*, 1983b).

Marked increases in chalcone synthase mRNA activity have also been observed in *P. vulgaris* hypocotyls during race:cultivar-specific interactions with the partially biotrophic fungus *Colletotrichum lindemuthianum*, causal agent of anthracnose (Bell *et al.*, 1984). In an incompatible interaction (host resistant) there is an early but localised increase in chalcone synthase mRNA activity correlated with the onset of phytoalexin accumulation and expression of hypersensitive resistance. In contrast, in a compatible interaction (host susceptible) there is no induction of mRNA activity in the early stages of infection, but rather a delayed widespread response at the onset of lesion formation associated with attempted lesion limitation.

These observations reveal a characteristic induction of chalcone synthase synthesis at the onset of phytoalexin accumulation in both elicitor-treated cells and infected tissues. The concomitant increases in activity levels of a number of enzymes of phytoalexin biosynthesis suggest that a similar mechanism of induction might operate for many if not all enzymes in the pathway.

In vivo labeling with ^{2}H from $^{2}H_2O$ coupled with analysis of the equilibrium distribution of enzyme activity in a density gradient has shown that elicitor also stimulates the rate of synthesis of phenylalanine ammonia-lyase, the first enzyme of general phenylpropanoid biosynthesis and chalcone isomerase, the second enzyme specific to the flavonoid/isoflavonoid branch pathway (Dixon and Lamb, 1979; Dixon *et al.*, 1983b; Lamb and Dixon, 1978; Lawton *et al.*, 1980). However, this technique measures the ratio of labeled to unlabeled enzyme (Lamb and Rubery, 1976) and therefore is not suitable for studies requiring short pulses of label for the detailed analysis of rapid regulatory responses.

To address the key question of whether there is a program of co-ordinate induction of enzyme synthesis governing expression of the phytoalexin defense response requires mono-

specific antisera to other enzymes of the pathway in addition to chalcone synthase. Previous *in vivo* labeling and *in vitro* translation studies using antiserum to phenylalanine ammonia-lyase from light-induced *Petroselinum hortense* (parsley) cell cultures suggested that there was a co-ordinate induction of phenylalanine ammonia-lyase and chalcone synthase synthesis in elicitor-treated *P. vulgaris* cells (Lawton *et al.*, 1983a, 1983b). However, subsequent studies (unpublished) have revealed that the antiserum to *P. hortense* phenylalanine ammonia-lyase (Schröder *et al.*, 1979) is not monospecific, at least with respect to *P. vulgaris* antigens, and that immunoprecipitation of contaminants might interfere with quantification, especially during *in vitro* synthesis, of [^{35}S]methionine incorporation into subunits of *P. vulgaris* phenylalanine ammonia-lyase (unpublished).

We have used monospecific antisera to phenylalanine ammonia-lyase and chalcone isomerase purified to homogeneity from elicitor-treated *P. vulgaris* cells (Robbins and Dixon, 1984; unpublished) together with the previously characterised monospecific antiserum to chalcone synthase (Lawton *et al.*, 1983a, 1983b; Schröder *et al.*, 1979) to compare the induction kinetics for synthesis of these three enzymes in both elicitor-treated cell cultures and infected hypocotyl tissue. We conclude that expression of the phytoalexin defense response in biologically stressed *P. vulgaris* cells characteristically involves co-ordinate induction of synthesis of phytoalexin biosynthetic enzymes.

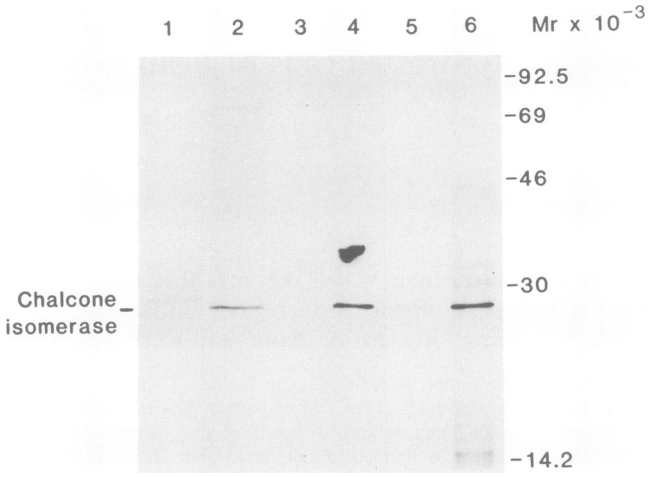

Fig. 1. Electrophoretic analysis of [^{35}S]methionine-labeled polypeptides immunoprecipitated with antiserum to chalcone isomerase purified to homogeneity from elicitor-treated *P. vulgaris* cell cultures. Indirect immunoprecipitation using the antiserum in conjunction with protein A-Sepharose was employed in all instances except **lane 1** which involved direct immunoprecipitation in the absence of protein A-Sepharose. **Lanes 1** and **2**: extracts from cells labeled *in vivo* with [^{35}S]methionine for 1 h immediately prior to harvesting 3 h after elicitor treatment; **lane 3**: *in vitro* translation products with polysomal RNA isolated from control, uninduced cells; **lane 4**: *in vitro* translation products with polysomal RNA isolated from cells 3 h after elicitor treatment; **lane 5**: *in vitro* translation products with polysomal RNA from site 1 of hypocotyls 79 h after infection with *C. lindemuthianum* spores of the incompatible race β; **lane 6**: *in vitro* translation products with polysomal RNA from site 1 of hypocotyls 150 h after infection with *C. lindemuthianum* spores of the compatible race γ.

Results

Immunoprecipitation of enzyme subunits

Immunoprecipitation of chalcone synthase subunits (mol. wt. 42 500) and phenylalanine ammonia-lyase subunits (mol. wt. 77 000) synthesised *in vivo* by elicitor-treated cells and by *in vitro* translation of RNA from elicitor-treated cells and infected hypocotyl tissue had been described previously (Bell *et al.*, 1984; Lawton *et al.*, 1983a, 1983b; unpublished). Treatment of extracts from elicitor-treated cells labeled *in vivo* with [^{35}S]methionine with antiserum to *P. vulgaris* chalcone isomerase failed to immunoprecipitate labeled chalcone isomerase subunits (Robbins and Dixon, 1984) (Figure 1). However, indirect immunoprecipitation using this antiserum in conjunction with protein A-Sepharose isolated a single [^{35}S]methionine-labeled polypeptide, mol. wt. 27 000 (Figure 1), the subunit mol. wt. of authentic chalcone isomerase from *P. vulgaris* (Robbins and Dixon, 1984). [^{35}S]-Methionine-labeled chalcone isomerase subunits, of identical mol. wt. to those synthesised *in vivo*, could also be isolated by a similar procedure from the products of mRNA translation *in vitro* using RNA isolated from elicitor-treated cells or infected hypocotyls (Figure 1).

Elicitor-induced changes in enzyme synthesis

Changes in the rate of enzyme synthesis in elicitor-treated cell cultures were measured by immunoprecipitation of labeled enzyme subunits from extracts of cells that had been exposed to [^{35}S]methionine for 30 min immediately prior to harvest. Elicitor caused marked but transient increases in the rates of synthesis of phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase from very low basal levels, concomitant with the phase of rapid increase in enzyme activity at the onset of phytoalexin accumulation (Figure 2A, B). The induction kinetics were broadly similar for all three enzymes with maximum rates of synthesis being attained between 3 and 4 h after elicitor treatment followed by a rapid decay to relatively low rates of synthesis. In the absence of elicitor there is a very weak induction of enzyme synthesis arising from experimental manipulation of the cell cultures at the start of the time-course.

Elicitor-induced changes in mRNA activity

Changes in mRNA activity were measured by immunoprecipitation of [^{35}S]methionine-labeled enzyme subunits synthesised *in vitro* by translation of polysomal or total RNA preparations from cells at various times after elicitor treatment. In both the polysomal and total RNA fractions from control, untreated cells only very low levels of activity of the mRNAs encoding phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase could be detected. Elicitor caused transient increases in the activities of these mRNAs in both polysomal and total RNA fractions with maximum mRNA activities 3 – 4 h after elicitation and subsequent rapid decay to relatively low levels (Figure 2C, D). For each enzyme the induction kinetics of mRNA activity in both polysomal and total RNA fractions closely corresponded to the kinetics for induction of enzyme synthesis *in vivo* especially in the early stages following elicitor treatment.

Induction of mRNA activity in infected hypocotyl tissue

In vitro translation of isolated polysomal RNA allows quantitative analysis of the pattern of protein synthesis without the interpretative problems inherent in an *in vivo* labeling approach (Schimke, 1973) which arise from possible changes in the uptake and compartmentalization of exogenous label as the interactions between host and fungus develop. In an incompatible interaction following application of spores of

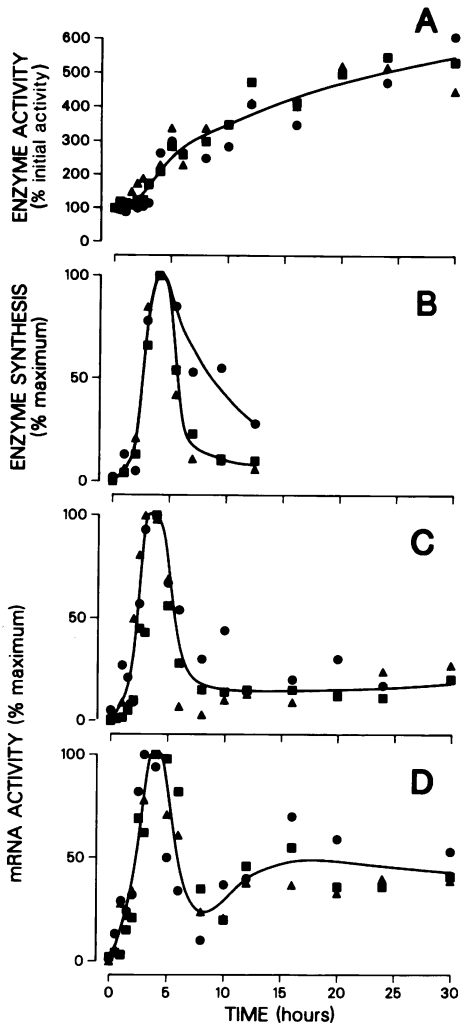

Fig. 2. Elicitor induction of phenylalanine ammonia-lyase (—●—), chalcone synthase (—▲—) and chalcone isomerase (—■—) in suspension-cultured *P. vulgaris* cells. **A**: enzyme activity; **B**: enzyme synthesis *in vivo*; **C**: translatable mRNA activity in the polysomal RNA fraction and **D**: translatable mRNA activity in the total cellular RNA fraction. Basal enzyme activities prior to elicitor treatment were: phenylalanine ammonia-lyase 4.9 μKat/kg protein; chalcone synthase 0.61 μKat/kg protein; chalcone isomerase 0.47 mKat/kg protein.

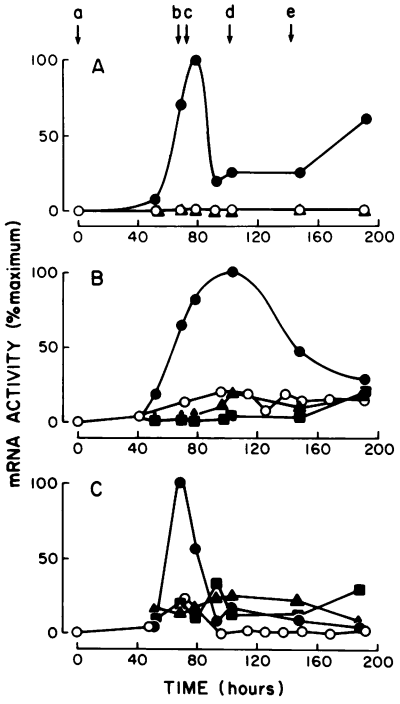

Fig. 3. Induction of polysomal mRNA activities encoding phenylalanine ammonia-lyase (**A**), chalcone synthase (**B**) and chalcone isomerase (**C**) in relation to phytoalexin accumulation and expression of hypersensitive resistance during an incompatible interaction (host resistant) between hypocotyls of *P. vulgaris* cultivar Kievitsboon Koekoek and *C. lindemuthianum* race β. mRNA activity was measured in directly infected tissue (site 1: —●—), in tissue laterally adjacent to the infected tissue (site 2: —▲—), in tissue beneath sites 1 and 2 (site 3: —■—) and in equivalent control, uninoculated hypocotyls (site 4: —○—). Arrows denote events in expression of hypersensitive resistance at site 1: a; spore inoculation; b: onset of hypersensitive flecking in a few sites; c: onset of phytoalexin accumulation; d: hypersensitive flecking apparent at most sites; d: very dense brown flecking at all sites; no visible changes in sites 2–4 throughout the time-course.

C. lindemuthianum race β to the unwounded surface of hypocotyls of *P. vulgaris* cultivar Kievitsboon Koekoek there were early but apparently localized increases in the mRNA activities encoding phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase correlated with the onset of phytoalexin accumulation and expression of hypersensitive resistance (Figure 3). Induction of mRNA activity was only observed in tissue at the sites of spore inoculation (site 1) and was not apparent in more distant tissue (sites 2 and 3).

In the compatible interaction with race γ there was no significant increase in mRNA activity above control levels during the early stages of infection equivalent to the phase in incompatible interactions of initial expression of hypersensitive resistance and increases in site 1 mRNA activity (Figure 4). Subsequently however, at the start of lesion development, there were marked increases in the activities of the mRNAs encoding phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase correlated with the onset of phytoalexin accumulation during attempted lesion limitation. Increases in mRNA activity were more pronounced and occurred slightly earlier in directly infected tissue at the site of spore inoculation. However, there were significant increases in the activities of the mRNAs encoding these three enzymes in tissue distant from the initial site of infection (sites 2 and 3). Induction of enzyme activity exhibited a similar temporal pattern to that for induction of mRNA activity (data not shown).

Discussion

The availability of monospecific antisera to phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase has allowed analysis of changes in the rate of synthesis of these enzymes in relation to expression of the phytoalexin defense response in *P. vulgaris*.

By three methods, marked but transient increases in the rates of synthesis of phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase could be observed during the initial phase of rapid increase in enzyme activity at the onset of phytoalexin accumulation. Within the limitations of the experimental techniques employed, there is a very close correlation between the kinetics for induction of synthesis of the three enzymes, especially in the initial stages following elicitor treatment, indicating that elicitor induces a co-ordinated stimulation of enzyme synthesis. Similarly, in infected hypocotyl tissue, co-ordinated induction of synthesis

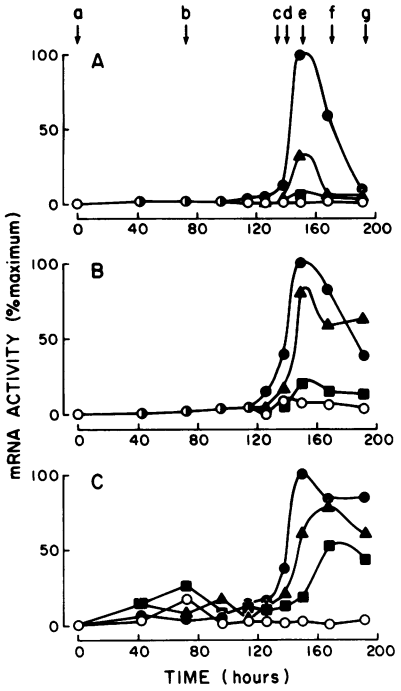

Fig. 4. Induction of polysomal mRNA activities encoding phenylalanine ammonia-lyase (**A**), chalcone synthase (**B**) and chalcone isomerase (**C**) in relation to phytoalexin accumulation and lesion development during a compatible interaction (host susceptible) between hypocotyls of *P. vulgaris* cultivar Kievitsboon Koekoek and *C. lindemuthianum* race γ. mRNA activity was measured in directly infected tissue (site 1: —●—), in tissue laterally adjacent to the infected tissue (site 2: —▲—), in tissue beneath sites 1 and 2 (site 3: —■—) and in equivalent control, uninoculated hypocotyls (site 4: —○—). Arrows denote events in lesion development at site 1: a: spore inoculation; b: no visible symptoms (cf. incompatible interaction); c: onset of symptom development at a few sites; d: onset of phytoalexin accumulation; e: pale to mid-brown lesions apparent at most sites; f: onset of water soaking and development of spreading lesions; g: extensive water soaking and spreading of lesions from site 1, some browning at site 2.

of phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase can be inferred from comparison of the spatial and temporal pattern of changes in the respective polysomal mRNA activities.

The data indicate that changes in the rate of enzyme synthesis are important components in the overall regulation of enzyme activity and flux through the biosynthetic pathway in both elicitor-treated cells and infected hypocotyls. However, it should be noted that post-translational regulation of each of these enzymes has been implicated in previous studies (Dixon and Robbins, 1984; Dixon *et al.*, 1982; Lawton *et al.*, 1980, 1983a) and concerted, reciprocal control over enzyme production and removal may provide for rapid, amplified and flexible responses of plant cells to biological stress (Lamb *et al.*, 1981; Paskin and Meyer, 1977).

The rapidity of induction of enzyme synthesis in elicitor-treated cells implies that this is not an indirect effect, but rather an early component in the causally related sequence of events between elicitor binding to a putative plant cell receptor (Larkin, 1981) and accumulation of phytoalexins. The close correlation between respective changes in enzyme synthesis measured *in vivo* and *in vitro* by translation of isolated RNA implies that elicitor stimulation of enzyme synthesis is largely a result of increased polysomal activity of the mRNAs encoding these enzymes. Furthermore, the marked increases in polysomal mRNA activities encoding phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase are increases as a proportion of total cellular mRNA activity, indicating that elicitor does not increase these polysomal mRNA activities by stimulation of selective recruitment from the total pool of cellular mRNA.

Using RNA blot hybridization with ^{32}P-labeled chalcone synthase cDNA sequences, rapid, marked, but transient increases in the amount of hybridizable chalcone synthase mRNA have been observed, consistent with the hypothesis that elicitor causes a rapid transient stimulation of transcription of chalcone synthase gene(s) (Cramer *et al.*, 1985; Ryder *et al.*, 1984, and unpublished). The highly co-ordinated increases in synthesis of phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase reported here strongly suggest that a similar mechanism of induction might operate for all three enzymes.

Materials and methods

Plant and fungal material

Seeds of *P. vulgaris* L. cultivar Kievitsboon Koekoek were germinated as previously described and grown at 25°C and 85% relative humidity under fluorescent light with a 16 h photoperiod (Rowell and Bailey, 1983). After 7 days, fully extended hypocotyls were excised 10 mm from the base and 20 mm below the cotyledons. The ends were sealed with molten paraffin wax and the hypocotyls incubated horizontally in humidified boxes at 16°C under the same light conditions.

C. lindemuthianum races β and γ were maintained, and sporulating cultures generated by u.v. irradiation, as previously described (Rowell and Bailey, 1983). Spores obtained after 6–8 days were suspended in distilled water, washed twice by centrifugation at 800 g_{av} for 3 min and finally resuspended in distilled water at a concentration of 5 x 10^5 spores/ml. Drops (5 μl) of this suspension were applied at 20 mm intervals along the upper surface of the hypocotyls. Lesion development was monitored visually and microscopically. Control hypocotyls were treated with sterile water in the absence of spores and incubated separately as above.

At appropriate intervals, 40–50 infected hypocotyls were collected, the terminal 5 mm portions removed and the remaining material excised by scalpel to yield collections of tissue from different regions of the hypocotyl in relation to the initial sites of spore inoculation. Thus the hypocotyls were bisected longitudinally in a horizontal plane and the upper half was then divided into two series of equal portions comprising respectively the tissue immediately underlying the initial sites of inoculation (site 1) and the residual portion of tissue between sites of inoculation (site 2). The lower half of the hypocotyl represented site 3 and control hypocotyls after removal of the wounded terminal portions represented site 4. Typically, at each time point between 150 and 210 samples of each type of site were collected giving ~4 g fresh weight of site 1 and site 2 material and 7 g fresh weight site 3 and site 4 material. Harvested tissue was frozen in liquid N_2 and stored at −70°C until required for enzyme assay or mRNA preparation.

Plant cell culture

Cells of *P. vulgaris* were grown as previously described (Dixon and Bendall, 1978), save that suspensions were cultured in total darkness. All experiments were conducted with 7–10 day old cultures, the medium of which exhibited a conductivity between 2.4–2.8 mhos.

Elicitor preparation

The source, maintenance and growth of *C. lindemuthianum* in batch liquid culture were as previously described (Bailey and Deverall, 1971). An elicitor preparation was obtained from the high mol. wt. fraction released from isolated mycelial walls by heat treatment (Anderson-Prouty and Albersheim, 1975). Elicitor was applied to cell cultures to give a final concentration of 60 μg glucose equivalents/ml.

Enzyme extraction and assay

Extraction and assay of phenylalanine ammonia-lyase (Lawton *et al.*, 1983a), chalcone synthase (Lawton *et al.*, 1983a) and chalcone isomerase (Dixon *et al.*, 1982) were as previously 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 s under the assay conditions.

Antisera

Monospecific antiserum to phenylalanine ammonia-lyase from elicitor-treated

cell cultures of *P. vulgaris* (unpublished), monospecific antiserum to chalcone isomerase from elicitor-treated cell cultures of *P. vulgaris* (Robbins and Dixon, 1984) and monospecific antiserum to chalcone synthase from light-induced cell cultures of *P. hortense* (Schröder *et al.*, 1979) had been prepared as described.

Protein synthesis in vivo

Labeling of enzyme *in vivo* by treatment of cell cultures with [^{35}S]methionine for 30 min immediately prior to harvest was as previously described (Lawton *et al.*, 1983a). Chalcone synthase and phenylalanine ammonia-lyase were separated from other labeled proteins in cell extracts by direct immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis (Lawton *et al.*, 1983a). Chalcone isomerase subunits were not immunoprecipitated by direct treatment with the appropriate antiserum (Robbins and Dixon, 1984) and therefore chalcone isomerase was separated from other labeled proteins in cell extracts by indirect immunoprecipitation with antiserum and protein A-Sepharose followed by SDS-polyacrylamide gel electrophoresis (Lawton *et al.*, 1983b; Robbins and Dixon, 1984). Enzyme subunits were located by fluorography and [^{35}S]methionine incorporation determined as previously described (Lawton *et al.*, 1983a). The rate of enzyme synthesis is defined as the incorporation of [^{35}S]methionine into enzyme subunits as a percentage of incorporation into total protein as previously described (Lawton *et al.*, 1983a). Temporal changes in the rate of synthesis in elicitor-treated cells are expressed relative to the rate of synthesis at maximal induction.

Isolation of RNA

Polysomal RNA for *in vitro* translation was isolated by a modification (Schröder *et al.*, 1976) of the method of Palmiter (1974). Total cellular RNA was isolated from cells homogenized directly in a phenol:0.1 M Tris (pH 9.0) emulsion (Haffner *et al.*, 1978). Further purification of the phenol-extracted total RNA was identical to that used for polysomal RNA (Palmiter, 1974). Extraction of RNA from elicitor-treated cells and infected hypocotyls of *P. vulgaris* has been previously characterised (Bell *et al.* 1984; Lawton *et al.*, 1983b). RNA was assayed spectrophotometrically at 260 nm.

Protein synthesis in vitro

Isolated polysomal RNA or total cellular RNA was translated *in vitro* in the presence of [^{35}S]methionine using an mRNA-dependent rabbit reticulocyte lysate translation system (Pelham and Jackson, 1976) and incorporation of [^{35}S]methionine into total protein was measured as described (Lawton *et al.*, 1983b). Chalcone isomerase, chalcone synthase and phenylalanine ammonia-lyase subunits were separated from other translation products by indirect immunoprecipitation with appropriate antiserum and protein A-Sepharose followed by SDS-polyacrylamide gel electrophoresis (Lawton *et al.*, 1983b; Robbins and Dixon, 1984). Enzyme subunits were located by fluorography and [^{35}S]methionine incorporation was determined as previously described (Lawton *et al.*, 1983a). 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 the mRNA activity at maximal induction.

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