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Rapid changes in gene expression in response to microbial elicitation

By R. A. Dixon[1], J. A. Bailey[2], J. N. Bell[3], G. P. Bolwell[1], C. L. Cramer[3], K. Edwards[4], M. A. M. S. Hamdan[1], C. J. Lamb[3], M. P. Robbins[1], T. B. Ryder[3] and W. Schuch[4]

[1] *Department of Biochemistry, Royal Holloway and Bedford New College, University of London, Egham Hill, Egham, Surrey TW20 0EX, U.K.*

[2] *Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, U.K.*

[3] *Plant Biology Laboratory, Salk Institute for Biological Studies, P.O. Box 85800, San Diego, California 92138, U.S.A.*

[4] *Imperial Chemical Industries plc, Corporate Biosciences Laboratory, The Heath, Runcorn, Cheshire WA7 4QE, U.K.*

Treatment of cell suspension cultures of French bean (*Phaseolus vulgaris*) with polysaccharide elicitor molecules from cell walls of the anthracnose fungus, *Colletotrichum lindemuthianum*, results in the rapid accumulation of isoflavonoid phytoalexins, deposition of wall-bound phenolic compounds and synthesis of hydroxyproline-rich glycoproteins. These changes are dependent upon a highly selective induction of gene products, including the enzymes L-phenylalanine ammonia-lyase, cytochrome P450-dependent cinnamic acid 4-hydroxylase, chalcone synthase, chalcone isomerase, prolyl hydroxylase and protein:arabinosyl transferase. Use of *in vivo* labelling, *in vitro* translation and RNA blot hybridization techniques has shown that these elicitor-mediated changes arise from rapid but transient induction of enzyme synthesis, resulting from the accumulation of specific mRNAs. Similar phenomena are observed in bean hypocotyls at the onset of phytoalexin synthesis in response to infection by incompatible and compatible races of *C. lindemuthianum*.

In bean, both L-phenylalanine ammonia-lyase and chalcone synthase are encoded by multigene families and, at the protein level, both exhibit subunit and intact enzyme polymorphism. A number of less than full-length phenylalanine ammonia-lyase copy DNAs containing identical open reading frames have been produced from mRNA from elicitor-induced bean cells. Analysis of phenylalanine ammonia-lyase genomic clones predicts the presence of enzyme forms of differing amino acid sequence. In cultured bean cells, elicitor differentially induces the two apparent phenylalanine ammonia-lyase iso-forms with the lowest K_m values.

In addition to transcriptional control of the appearance of specific gene products, post-translational processes may result in increased subunit polymorphism for phenylalanine ammonia-lyase, and in the activation of chalcone isomerase. Changes in endogenous phenylpropanoid intermediate pools may signal the rapid removal of phenylalanine ammonia-lyase activity, in addition to exerting less specific inhibitory effects on the formation and/or activity of the mRNAs encoding phenylalanine ammonia-lyase and other phytoalexin biosynthetic enzymes.

1. Introduction

In plants, the expression of disease resistance towards microbial pathogens often involves active, rapidly induced responses resulting in the accumulation of products that are either toxic to pathogens or that physically arrest their further growth in the host. Such active defence

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responses, which may also be induced by treatment of plant cells with molecules isolated from pathogen cell walls or culture fluids (elicitors), are now known to result from the rapid differential induction of a highly selective group of defence-related host genes. As with other phenomena of differential gene expression in plants, the study of induced disease resistance responses at the molecular level encompasses stimulus reception (elicitor receptors), intracellular signalling mechanisms, control of host gene expression and processing and function of induced gene products. We are still largely ignorant of the details of most of these processes. This article reviews recent work on the induction of resistance-related gene products in French bean (*Phaseolus vulgaris*) cells in response to infection by the anthracnose fungus *Colletotrichum lindemuthianum* and to elicitors isolated from this organism. More detailed reviews of the enzymological and plant pathological aspects of this work have appeared elsewhere (Dixon *et al.* 1983*a*; Dixon 1986).

2. The bean–*Colletotrichum* interaction as a model system for studies on the differential expression of genes related to plant disease resistance

Colletotrichum lindemuthianum, the causal agent of anthracnose disease of bean, is a hemibiotrophic fungus that exists as a number of physiological races, each of which gives differential responses on different host cultivars. The resistant interaction results in rapid hypersensitive death of the first infected cell at around the time of penetration of its cell wall, followed by the accumulation of isoflavonoid phytoalexins (low M_r, antimicrobial compounds produced by the plant) in a small area localized around the site of infection. In contrast, in susceptible interactions the first infected cell initially remains alive, and primary intracellular hyphae grow out from it; after a period of biotrophy of less than 24 h, the cytoplasm of infected cells gradually degenerates and, 48 h after their infection, cells appear dead. The formation of secondary hyphae is then associated with death of cells in advance of infection and the delayed accumulation of low concentrations of phytoalexins per threatened cell. These different patterns have been defined in terms of both phytoalexin production and cytology (Bailey & Deverall 1971; O'Connell *et al.* 1985). In most race–cultivar specific interactions, avirulence (i.e. triggering of resistance) is believed to result from the interaction of a single dominant pathogen gene product with the product of a corresponding host resistance gene (Ellingboe 1982). Conversely, susceptibility is seen as reflecting the absence of the initial recognitional event that triggers the hypersensitive response.

The induction of phytoalexins can be induced in suspension-cultured bean cells by exposure to elicitor molecules heat-released from the cell walls of *C. lindemuthianum* (Dixon & Lamb 1979). Use of these components, which are polysaccharides containing mannose, glucose and galactose (Hamdan & Dixon 1986*a*), does not, however, reproduce the race-specific elicitation of phytoalexins observed in the intact plant–pathogen system. Nevertheless, comparative studies of the nature, extent and timing of induced metabolic changes in the natural and *in vitro* systems (Robbins *et al.* 1985; Whitehead *et al.* 1982) indicates that sufficient similarities exist to warrant the use of elicitor-induced cell cultures as a simplified model system for the study of the basic mechanisms underlying gene expression associated with active defence.

The main features of the bean–*Colletotrichum* system that make it suitable for detailed molecular analysis are: (1) its well-defined biology and physiology (Bailey 1982; O'Connell

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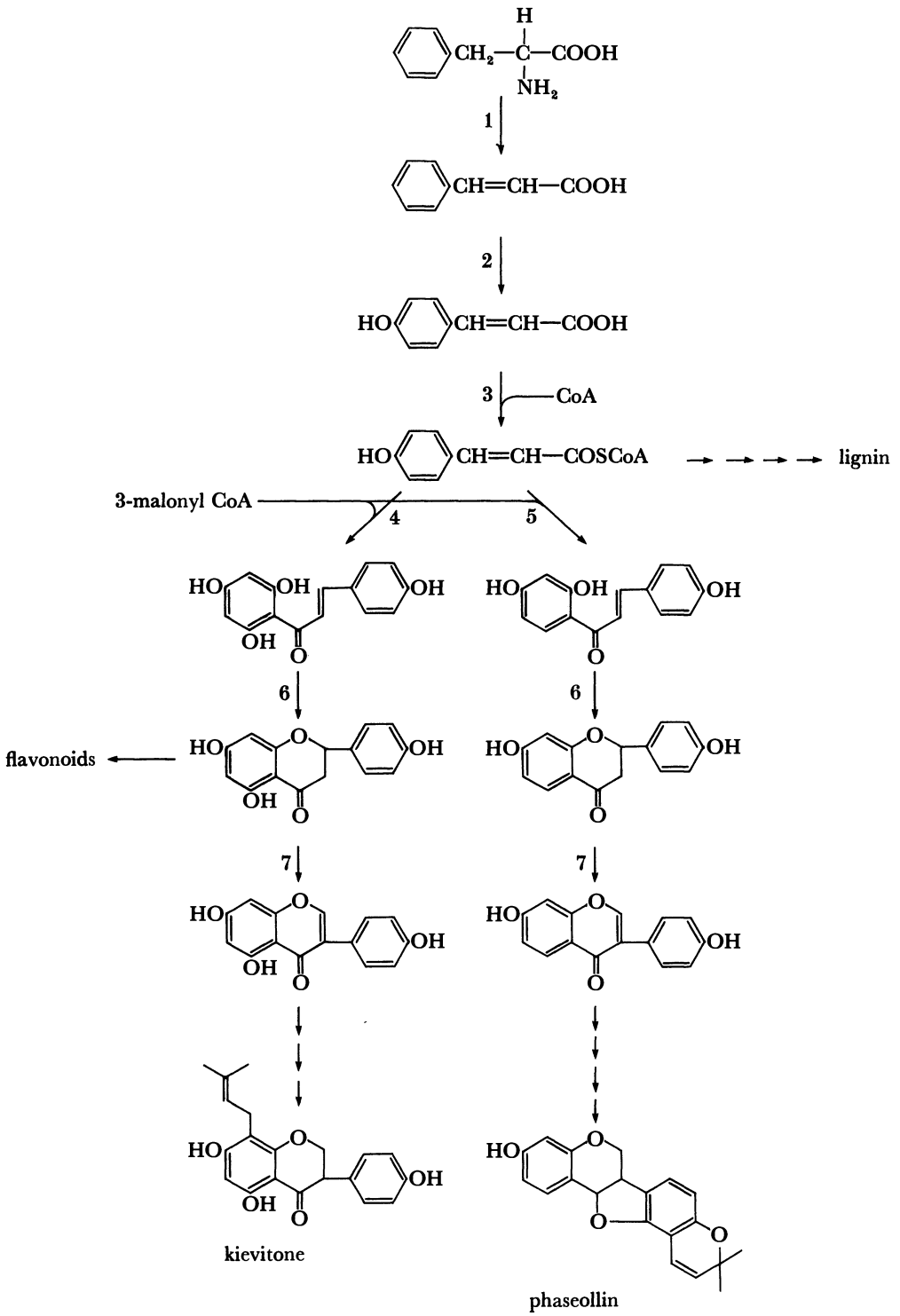

FIGURE 1. Biosynthesis of isoflavonoid phytoalexins in bean. The enzymes involved are: 1, L-phenylalanine ammonia-lyase; 2, cinnamic acid 4-hydroxylase; 3, 4-coumarate:CoA ligase; 4, chalcone synthase; 5, 6′-deoxychalcone synthase; 6, chalcone isomerase; 7, isoflavone synthetase.

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et al. 1985); (2) the clear genetic basis for the distinction between resistant and susceptible interactions; (3) the rapidity and selectivity of elicitor-induced gene expression; (4) the involvement of several metabolically unrelated pathways leading to gene products involved in the active defence response; (5) the reasonably well-defined enzymology of parts of the induced pathways; (6) the involvement of certain key regulatory enzymes, which exist in multiple forms. These iso-forms may arise from the expression of multigene families, may have different kinetic properties and/or functions and may be differentially induced both temporally and in response to different external stimuli.

3. Nature and timing of induced metabolic changes associated with active defence

The most studied active defence response in plants is the accumulation of phytoalexins, whose chemical class depends on the host-plant species, not the invading pathogen. In bean, these compounds are isoflavonoids derived from the amino acid L-phenylalanine via a metabolic pathway whose early stages are also common to the synthesis of hydroxycinnamic acids, lignin and flavonoid pigments (figure 1). The phytoalexins phaseollin and kievitone are the products of parallel but clearly differentiated pathways characterized by the presence or absence of a hydroxyl group at position 5 on the A-ring of the isoflavonoid precursor. That these two pathways are under differential regulation is apparent from the much more rapid induction of kievitone than of phaseollin in bean cotyledons (Whitehead *et al.* 1982) and cell suspension cultures (Robbins *et al.* 1985) (table 1). Related phenolic compounds (hydroxycinnamic acids and flavonol-like material) accumulate in the cell walls of elicitor-treated bean cultures, and are responsible for the rapid browning that generally accompanies phytoalexin accumulation (Dixon & Bendall 1978). These components are associated with both the

Table 1. Appearance of defence-related products in elicitor-treated bean cell cultures[a]

	product	units per gram fresh mass	time (hours) to maximum nduced level or activity	basal level	maximum induced level
phytoalexins	kievitone	nmol	12	n.d.[c]	42
	phaseollin	nmol	48	n.d.	537
wall-bound phenolics	in hemicellulosic fraction	A_{310}	24	0.5	21
	in cellulosic fraction	A_{310}	> 24	4.5	> 22
peptide hydroxyproline	in endomembrane system	µg[b]	4	4	6.8
	in cell wall	mg	6	0.1	0.29
chitinase		µkat	48	0.135	0.195

[a] Cell suspension cultures of *Phaseolus vulgaris* c.v. Immuna were treated with elicitor (25 µg glucose equivalents ml^{-1}) heat-released from the cell walls of *Colletotrichum lindemuthianum*.
[b] Units per milligram membrane protein.
[c] Not detected.

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cellulosic and hemicellulosic fractions of the wall (Bolwell *et al.* 1985*b*). It has been speculated that changes in host cell wall composition may provide physical–chemical barriers that localize the pathogen. In addition, hydroxyproline-rich glycoproteins also accumulate in the wall as part of the coordinated defence response (Bolwell *et al.* 1985*b*), and these may function either to strengthen the wall further or to act as bacterial agglutinins (Leach *et al.* 1982). The production of peptide hydroxyproline in the endomembrane system, followed by deposition in the wall, precedes the accumulation of isoflavonoid phytoalexins in elicitor-treated bean cultures (table 1).

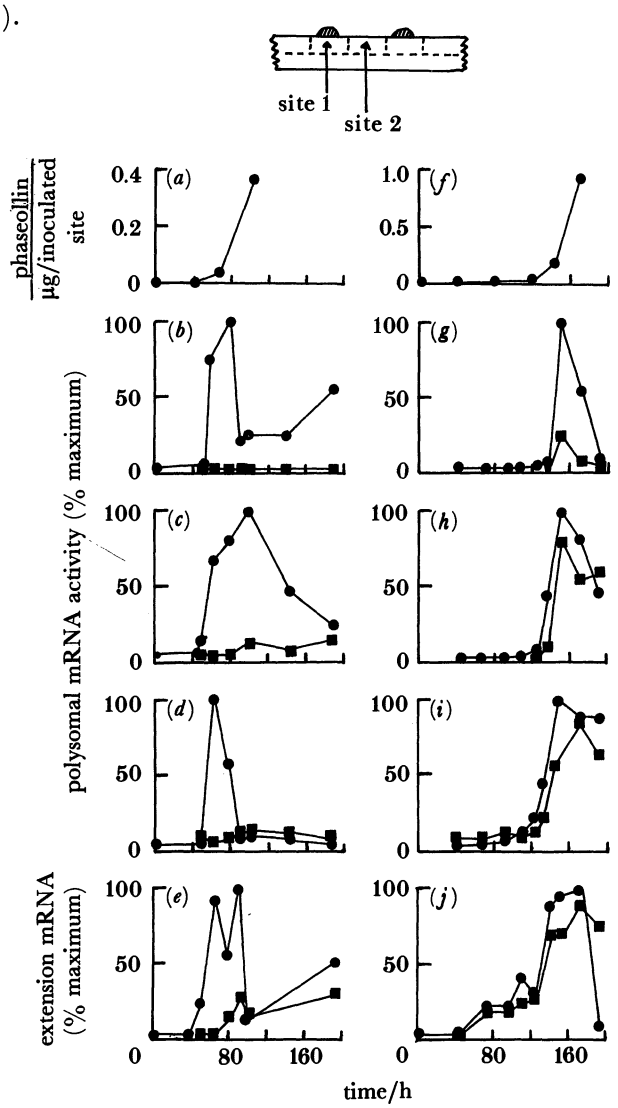

FIGURE 2. Induction of messenger RNAs in resistant and susceptible interactions of bean (cv. Kievitsboon koekoek) with *Colletotrichum lindemuthianum*. (*a–e*) hypersensitive resistant response (*C. lindemuthianum* race β); (*f–j*) susceptible interaction (*C. lindemuthianum* race γ). (*a,f*) Phytoalexin (phaseollin) levels per inoculated site; (*b,g*) phenylalanine ammonia-lyase mRNA activity; (*c,h*) chalcone synthase mRNA activity; (*d,i*) chalcone isomerase mRNA activity; (*e,j*) 2.7 kb hydroxyproline-rich glycoprotein (HRGP) mRNA level. mRNA activities were determined by *in vitro* translation of total polysomal RNA followed by specific immunoprecipitation. HRGP mRNA was determined by blot hybridization to a ^{32}P-labelled tomato HRGP genomic sequence. Tissue was analysed from sites directly inoculated with *Colletotrichum* spore suspension (site 1, ●) or from sites adjacent to the inoculated areas (site 2, ■).

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The accumulation of phenolics, isoflavonoids and hydroxyproline-rich glycoproteins requires the action of a large number of biosynthetic enzymes, whose induction is considered in the following section. In addition to such multienzyme pathways, some single-gene products may also be components of the induced defence response; a typical example is the enzyme chitinase, whose induction may be related to the production of ethylene (Boller *et al.* 1983) and which may act in the degradation of fungal cell walls. Chitinase undergoes a small but significant induction in elicitor-treated and infected bean cells (M. P. Robbins, unpublished; Bell *et al.* 1985*a*).

It should be noted that the metabolic changes outlined in table 1 begin very rapidly after exposure of cultured bean cells to elicitor. In infected bean hypocotyls, changes associated with phytoalexin accumulation and hydroxyproline-rich glycoprotein synthesis are similarly coordinated, but occur at different times, related to the timing of the hypersensitive response or lesion formation, in resistant and susceptible interactions (Cramer *et al.* 1985*a*; Showalter *et al.* 1985) (see figure 2).

4. Differential induction of gene products in response to microbial elicitation

(*a*) *Selectivity of enzyme induction and mRNA appearance*

Two-dimensional isoelectric focusing and sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoretic analysis of polypeptides, synthesized either *in vivo* or *in vitro* from mRNA, from elicitor-treated bean cell cultures reveals the specific induction of a limited number of polypeptides and a similar number whose mRNA activities are specifically depressed (Cramer *et al.* 1985*b*; Dixon *et al.* 1986*a*). Most polypeptides remain unaffected by elicitor treatment. At the enzyme activity level, 12 enzymes in areas of metabolism closely related but peripheral to the phytoalexin pathway have been shown to be unaffected by elicitor in cell suspension cultures or by infection in bean hypocotyls, whereas the increased appearance of others may be prevented by elicitor treatment (Robbins *et al.* 1985; Bolwell *et al.* 1985*b*). The response of both cell cultures and infected hypocotyls is, however, characterized by the striking induction of enzymes directly involved in the biosynthesis of phenolic compounds, isoflavonoid phytoalexins and hydroxyproline-rich glycoproteins.

(*b*) *Enzymes of isoflavonoid phytoalexin biosynthesis*

The enzymology of the isoflavonoid phytoalexin pathway in bean is reasonably well elucidated as far as the chalcone isomerase reaction (figure 1). These enzymes are, however, also involved in the synthesis of wall-bound phenolic components in bean. The later reactions specific for isoflavonoid formation have been investigated only in soybean (*Glycine max*); some of the enzymes are cytochrome P450-linked, and appear to be very unstable *in vitro* (Hagmann *et al.* 1984; Hagmann & Grisebach 1984).

L-Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyses the first committed reaction in the biosynthesis of phenylpropanoid compounds from L-phenylalanine. There is much current interest in the regulation of this enzyme, as it may be induced by a wide range of stimuli including light, wounding, plant growth regulators, dilution of cell cultures, elicitors and microbial attack (Jones 1984). However, the end-products of the phenylpropanoid pathway that accumulate depend on the nature of the applied stimulus, and in addition to PAL

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induction also reflect concomitant induction of later regulatory enzymes on specific branch pathways; for example, in bean, light induces accumulation of flavonoids, plant growth regulators stimulate lignification and microbial stimuli induce isoflavonoid phytoalexins.

PAL from most sources appears to be a tetramer possessing two dehydroalanine-containing active sites per molecule (Jones 1984). The subunit M_r for the bean enzyme is 77000, although partial degradation products (which retain some catalytic activity) of M_r 70000 and 53000 are readily formed *in vitro* during purification, and *in vivo* during normal subunit turnover (Bolwell *et al.* 1986*b*). Polypeptides of these relative molecular masses are also obtained after immunoprecipitation of PAL subunits synthesized *in vitro* from mRNA. Chromatofocusing analysis reveals the presence of four distinct active PAL tetramer fractions, of differing p*I* but exhibiting similar subunit M_r patterns; PAL forms separated by this procedure exhibit normal Michaelis–Menten kinetics, with decreasing K_m values in order of increasing p*I*, whereas purified PAL preparations not subjected to separation on the basis of p*I* exhibit apparent negative rate cooperativity with respect to the substrate L-phenylalanine. This suggests that the complex kinetics reported for PAL may reflect the combined activities of a number of different forms of the enzyme with differing K_m values. Elicitor treatment of bean cell suspension cultures results in the differential appearance of the two higher p*I*, lower K_m PAL forms as revealed by chromatofocusing analysis (Bolwell *et al.* 1985*a*).

Density labelling studies with ^2H from ^2H_2O, followed by analysis of the equilibrium distribution of enzyme activity on high resolution KBr density gradients, demonstrated that the rapid, transient induction of PAL activity in elicitor-treated bean cell suspension cultures results from a transient increase in the rate constant for *de novo* synthesis accompanied, in experiments where high elicitor concentrations were used, by an inhibition of the removal of active enzyme (Lawton *et al.* 1980). By the time of attainment of maximum enzyme activity, the synthetic rate had declined and the normal rate of turnover was restored. The rapid, *de novo* synthesis of PAL in elicitor-treated bean cultures has subsequently been confirmed by *in vivo* [^{35}S]methionine labelling experiments, and the increased synthetic rate has been shown to result from increased translatable mRNA activity (in the total, polysomal and poly(A)$^+$ RNA fractions), as measured by *in vitro* translation in a rabbit reticulocyte lysate cell-free system (Lawton *et al.* 1983*a*, *b*; Cramer *et al.* 1985*a*). The kinetics of induction of the enzymic and mRNA activities for PAL and other defence-related enzymes in bean cell cultures are summarized in table 2, and the relation of changes in mRNA activity to phytoalexin accumulation in compatible and incompatible interactions of bean with *Colletotrichum* detailed in figure 2.

The elicitor-mediated increases in PAL mRNA activity result from increased PAL gene transcription. Thus a rapid transient induction of PAL mRNA level has been shown by Northern blot hybridization to a PAL copy DNA (cDNA) probe (Edwards *et al.* 1985), and by solution hybridization to an intron/exon boundary sequence from a bean PAL genomic clone followed by analysis of the fragment protected from S1 nuclease digestion (G. P. Bolwell, K. Edwards & R. A. Dixon, unpublished). Thiouridine-labelling experiments have revealed that elicitor-induced PAL mRNA is in the newly synthesized RNA fraction separable by organomercurial affinity chromatography (Edwards *et al.* 1985), and increased amounts of newly synthesized PAL transcripts have been observed in run-off analyses with nuclei from elicitor-treated compared with control bean cells (G. P. Bolwell, K. Edwards & R. A. Dixon, unpublished; Lawton & Lamb 1986).

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TABLE 2. CHANGES IN GENE PRODUCTS AND THEIR CORRESPONDING mRNAs IN ELICITOR-TREATED BEAN CELL CULTURES[a]

		enzyme activity (or protein level)[c]				mRNA activity (or amount)[d]			
		time (hours) to maximum induced value		fold increase		time (hours) to maximum induced value		fold increase	
area of metabolism	product[b]								
phytoalexin and phenolic synthesis	phenylalanine ammonia-lyase	8–10	(8–10)	4–6	(4)	2–4	(2–4)	5.5	(5)
	cinnamic acid 4-hydroxylase	6	—	2.9	—	—	—	—	—
	cytochrome P450	—	(6)	—	(2)	2	(n.d.)[e]	3	(n.d.)
	chalcone synthase	8	(n.d.)	8	(n.d.)	2.4	(4)	5	(10)
	chalcone isomerase	16	(12)	3.3	(6)	6	(n.d.)	10	(n.d.)
hydroxyproline-rich glycoprotein synthesis	extensin	—	(n.d.)	—	(n.d.)	n.d.	(> 35)	n.d.	(~100)
	M_r 42500 protein	4	(n.d.)	4	(n.d.)	n.d.	(n.d.)	n.d.	(n.d.)
	prolyl hydroxylase[f]	6	(n.d.)	3.8	(n.d.)	2	(n.d.)	5	(n.d.)
	protein arabinosyl transferase	4	(n.d.)	3	(n.d.)	n.d.	(n.d.)	n.d.	(n.d.)

[a] Cell suspension cultures of *Phaseolus vulgaris* were treated with elicitor (25 μg glucose equivalents ml^{-1}) heat-released from the cell walls of *Colletotrichum lindemuthianum*.

[b] See text for details.

[c] Figures in parentheses are the levels of immunodetectable protein measured by immune blotting techniques.

[d] mRNA activity was measured by *in vitro* translation analysis, mRNA amount (figures in parentheses) by RNA blot hybridization.

[e] Not determined.

[f] A later increase in prolyl hydroxylase activity (peak value more than 24 h) may relate to the changes in extensin synthesis.

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The charge iso-forms of active PAL observed by chromatofocusing reflect a complex charge polymorphism at the subunit level. At least 11 subunit forms, of identical M_r but differing pI in the range 4.8–5.5, are consistently observed on two-dimensional isoelectric focusing and SDS–polyacrylamide gel electrophoretic analysis of immunoprecipitates from elicitor-treated bean cells labelled with [^{35}S]methionine *in vivo* (Bolwell *et al.* 1985*a*). It is not easy to assess the extent to which post-translational changes (either real or artefactual) may be involved in generating this polymorphism. Up to six subunit charge forms are observed after *in vitro* translation of bean cell culture mRNA although, as PAL accounts for only about 0.02% of total mRNA and also translates poorly *in vitro* relative to other mRNAs, poor signal strengths may mean that this is an underestimate. Analysis of five PAL cDNA clones isolated from a pAT153 library of sequences complementary to elicitor-induced bean mRNA has failed to reveal differences in open-reading-frame sequences that could generate the observed polymorphism. However, none of these clones was full-length, and it has been observed that partial degradation products of PAL exhibit greatly reduced subunit polymorphism (11 forms of M_r 77000 ⟶ 2 forms of M_r 70000 ⟶ 1 form of M_r 53000) (Bolwell *et al.* 1986*b*). This finding might explain the identical cDNA sequences if N-terminal fragments are lost during the partial degradation of PAL. The PAL cDNAs contained identical sequences in their 3′-untranslated regions, but the lengths of sequence between the translation stop signal and the polyadenylation site differed in three of the clones, yielding total 3′-untranslated region lengths (up to the start of the poly(A) tails) of from 132 to 191 base pairs. Recently, a PAL cDNA clone has been isolated from a λgt11 library and shown to contain sequence differences in the regions conserved in the five clones from the pAT153 library (C. L. Cramer, M. H. Walter & C. J. Lamb, unpublished).

Southern blot hybridization analysis indicates that bean PAL is encoded by a multigene family, and at least four distinct PAL genes have been isolated from λgt WES and λ1059 bean genomic libraries; the amount of hybridization on Southern blots suggests that some members of the family may be present in the bean genome in up to five copies. Sequence analysis has revealed significant differences in both 3′-untranslated regions and open reading frames, and gene-specific probes are now being used to study the differential transcription of PAL genes in response to elicitation and other environmental signals. The gene from the λgt WES library analysed in most detail (gPAL2, 5.5 kilobase pairs (kb)) contains a 1.7 kb intron approximately 550 base pairs (bp) downstream from the transcription start, and a 1.2 kb 5′ upstream region of this gene is now under analysis in relation to sequences involved in the regulation of PAL transcription. The 5′-end of the incomplete pPAL5 cDNA clone encodes a hydrophobic peptide sequence (9 leucines, 3 valines and 2 alanines in a total of 23 residues) that is not fully conserved in the corresponding region of the gPAL2 genomic clone. The relation between PAL genes, subunits and active tetramers is currently under investigation.

Cinnamic acid 4-hydroxylase (CA4H; EC 1.14.13.11), the second enzyme of the central phenylpropanoid pathway, is a cytochrome P450-dependent mixed function oxygenase. Use of an anti-(rat P450) monoclonal antibody, which recognizes a highly conserved epitope, in Western blot and immunoprecipitation experiments has identified an M_r 48000 polypeptide that is synthesized on membrane-bound polysomes and whose mRNA activity, rate of synthesis and amount are rapidly induced by elicitor coordinately with the increase in CA4H activity (Bolwell & Dixon 1986) (table 2). As cytochrome P450 is also apparently involved in several of the later enzymic reactions specific for isoflavonoid synthesis, the isolation and

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characterization of bean cytochrome P450 cDNAs will provide an important basis for the study of the induction of these later reactions whose enzymology is at present poorly understood.

Chalcone synthase (CHS) catalyses the formation of 2′,4,4′,6′-tetrahydroxychalcone from 4-coumaroyl CoA and three molecules of malonyl CoA (figure 1). This is the first committed step in the biosynthesis of flavonoids/isoflavonoids from the central phenylpropanoid pathway. CHS is currently of great interest at the molecular genetic level, especially in relation to its light induction and resultant use as a model for the study of light-induced promoter sequences (Kaulen *et al.* 1986). The enzyme from bean is a dimer, whose subunits appear on one-dimensional SDS–polyacrylamide gel electrophoresis (PAGE) as a doublet of approximate M_r 42000 to 43000. Its activity is rapidly but transiently induced in response to elicitor in bean cell suspension cultures, and after infection by *Colletotrichum* in bean hypocotyls (table 2, figure 2). As with PAL, induction of CHS is associated with an increased rate of *de novo* synthesis resulting from increased gene transcription (Lawton *et al.* 1983*a*, *b*; Ryder *et al.* 1984; Lawton & Lamb 1986). Enzyme subunits exhibit identical charge polymorphism (up to ten subunits of differing p*I*) when synthesized both *in vivo* or *in vitro* from mRNA (Bell *et al.* 1986*b*; Hamdan & Dixon 1986*b*; Ryder *et al.* 1986), and the active enzyme likewise exhibits charge polymorphism on chromatofocusing analysis (Hamdan & Dixon 1986*b*). CHS is one of the most abundant mRNA species in elicitor-induced bean cells, accounting for at least 1% of total mRNA activity.

A bean CHS cDNA clone was initially isolated from a pBR325 bean cDNA library by probing with a clone complementary to a parsley (*Petroselinum hortense*) CHS sequence (Ryder *et al.* 1984). More recently, a number of different CHS genes have been isolated from a bean genomic library, some of which appear to be linked on the same λ clones. The production of gene-specific probes, based on 3′-untranslated region sequences, has allowed the direct confirmation of the activation of several specific CHS genes by elicitor and infection (Ryder *et al.* 1986).

The existence of a separate 6′-deoxy CHS, necessary for the formation of 5-deoxy isoflavonoid derivatives such as phaseollin in bean, has been predicted on the basis of [^{13}C]acetate-labelling experiments (Dewick *et al.* 1982), but this activity has not yet been shown in a cell-free system.

Bean chalcone isomerase (CHI; EC 5.5.1.6) catalyses the conversion of 2′,4′,4′-tri- and 2′,4,4′,6′-tetrahydroxy chalcones to their corresponding (−) flavanones. It is a monomeric enzyme, of subunit M_r 27500, and does not exhibit charge polymorphism (Robbins & Dixon 1984; Hamdan & Dixon 1986*b*). As with the preceding enzymes, induction of CHI in response to elicitor involves *de novo* synthesis, resulting from increased mRNA appearance (Cramer *et al.* 1985*a*), although density labelling and immunoblotting experiments have revealed that a proportion of the observed activity increase results from enzyme activation (Dixon *et al.* 1983*c*; Robbins & Dixon 1984). It is not yet clear whether activation is an early direct consequence of elicitor action, or whether it results from the formation of cinnamic acid by the PAL reaction; when added exogenously, cinnamic acid can induce CHI enzyme activity (Gerrish *et al.* 1985) while at the same time stimulating the loss of CHI mRNA activity (see § 5). Elicitor-mediated CHI mRNA activity may be induced coordinately with corresponding changes in PAL and CHS mRNAs (Cramer *et al.* 1985*a*), although in some bean cell culture lines its maximum level is reached later (table 2). In infected bean hypocotyls, changes in CHI mRNA activity coincide with those of PAL and CHS at the onset of the phytoalexin response (figure 2). CHI

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mRNA occurs in the newly synthesized RNA fraction from elicitor-treated bean cell cultures, indicating that its increased appearance is the result of activated gene transcription (Cramer *et al.* 1985*b*).

(*c*) *Hydroxyproline-rich glycoprotein synthesis*

Hydroxyproline-rich glycoproteins (HRGPs) are initially synthesized as proline-rich polypeptide precursors, whose proline residues are post-translationally hydroxylated in the endomembrane system by the enzyme prolyl hydroxylase (peptide proline:2-oxoglutarate dioxygenase; EC 1.14.11.12). Further modification leads to the glycosylation of a significant number of hydroxylated amino acids, predominantly with arabinose-rich side chains.

mRNAs encoding the HRGPs of the extensin family accumulate in *Colletotrichum*-infected bean hypocotyls with approximately similar kinetics to the appearance of the mRNAs encoding the phytoalexin biosynthetic enzymes PAL and CHS (figure 2). This has been deduced from the use of a tomato extensin genomic clone in Northern hybridization analyses of mRNA from infection sites from compatible and incompatible interactions (Showalter *et al.* 1985). Three mRNA species, of 1.6, 2.7 and approx. 5.6 kb, were induced in the bean hypocotyls, and in cell suspension cultures exposed to elicitor from *C. lindemuthianum*.

In cell suspension cultures, extensin mRNA appearance was delayed compared with the appearance of mRNAs encoding phytoalexin biosynthetic enzymes (Showalter *et al.* 1985) and nuclear transcript run-off analysis has shown a lag of 2 h before stimulation of HRGP gene transcription (Lawton & Lamb 1986). However, an arabinosylated hydroxyproline-rich glycoprotein of M_r 42500 is rapidly induced in elicitor-treated bean cultures, and its appearance coordinates with the transient induction of prolyl hydroxylase and protein:arabinosyl transferase extractable activities (Bolwell 1984; Bolwell *et al.* 1985*b*). In unelicited control cultures, the transfer of arabinose to biopolymers is predominantly associated with the arabinan synthetase reaction of cell wall biosynthesis; on elicitation this reaction appears to stop, and arabinose is transferred predominantly on to protein, in particular the M_r 42500 polypeptide (Bolwell 1984). A later increase in prolyl hydroxylase activity in elicited cells may be related to the observed appearance of extensin mRNA.

Bean prolyl hydroxylase has been purified to homogeneity (Bolwell *et al.* 1985*c*) and a polyclonal antiserum prepared against it (Bolwell & Dixon 1986). The enzyme has a subunit M_r of 65000, is loosely associated with smooth endomembranes, and shows many similarities to the well-characterized mammalian enzyme. *In vivo* labelling and *in vitro* translation experiments have demonstrated that elicitor rapidly induces the *de novo* synthesis of the M_r 65000 subunit; an associated, antigenically related M_r 60000 subunit is also immunoprecipitated, but its rate of synthesis is not stimulated by elicitor (Bolwell & Dixon 1986).

(*d*) *Others*

The enzyme chitinase (EC 3.2.1.14) has previously been studied in bean in relation to its induction by ethylene (Boller *et al.* 1983), and the chitinase gene has now been cloned and sequenced (R. Broglie, unpublished). Both chitinase and endo-1,3-β-D-glucanase activities are induced in bean cells in response to *Colletotrichum* elicitor, although the overall extent of their induction appears less than that of the phytoalexin biosynthetic enzymes (M. P. Robbins, unpublished; Bell *et al.* 1986*a*). A direct involvement of ethylene in disease resistance responses of bean has not been established.

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The exact nature of the wall-bound phenolic material that accumulates in elicitor-treated bean cells is not known, although esterified hydroxycinnamic acids and flavonoid-derived compounds may in part be involved (Dixon & Bendall 1978). That the synthesis of lignin precursors such as coniferyl alcohol may be induced (either as free or, ultimately, as wall-bound components) is suggested by the recent demonstration of the induction by elicitor of coniferyl alcohol dehydrogenase (EC 1.1.1.1) mRNA activity (C. Grand, M. H. Walter & C. J. Lamb, unpublished). Other enzymes of lignin synthesis (e.g. caffeic acid *O*-methyltransferase and peroxidase) are not induced by elicitor (Robbins *et al.* 1985), whereas the activity of 4-coumarate hydroxylase, which increases in non-elicited cultures, remains at basal levels in elicited cells (Bolwell *et al.* 1985*b*).

(*e*) *Temporal and spatial coordination of induced responses*

It is evident from the preceding sections that elicitation or infection result in the coordinated appearance of several gene products and their corresponding mRNAs. The pattern of coordination of changes in expression of phenylpropanoid pathway components in elicited bean cells is different from that observed during the light-induction of flavonoid accumulation in parsley cells, where the enzymes of the central phenylpropanoid pathway (PAL, CA4H, 4-coumarate:CoA ligase), are induced together but earlier than the enzymes of the flavonoid branch pathway (CHS, CHI and subsequent reactions), which themselves form a coordinately induced group (Hahlbrock & Grisebach 1979). In bean cell suspension cultures, increased appearance of several mRNAs (e.g. PAL, CHS, cytochrome P450, prolyl hydroxylase) occurs extremely rapidly after exposure to elicitor; the gene activation resulting in their formation is presumably therefore a response to an early signal associated with the initial processes triggered by elicitor. Other genes, for example those encoding enzymes specific for 5-deoxyisoflavonoid formation, may be switched on later in response to different, secondary signals. The mechanism of signal transduction during elicitation is at present unknown; it is hoped that an understanding of this may arise from analysis of promoter regions of elicitor-induced genes followed by the identification of possible sequence-specific gene-activation factors, in addition to attempts to isolate elicitor receptors and intracellular response couplers.

Although the appearances of PAL and CHS mRNAs follow similar kinetics in elicitor-treated bean cells, their induction may, under some circumstances, be uncoupled. Fractions of *Colletotrichum* culture filtrate elicitor, separated by chromatography on concanavalin A Sepharose, can induce CHS in the absence of PAL and CHI induction, whereas the size-fractionated elicitor preparation from which they were purified induces all three enzymes (Dixon *et al.* 1986*b*).

In *Colletotrichum*-infected bean hypocotyls there is a distinct temporal differentiation between expression of defence-related genes in the incompatible and compatible interaction (figure 2). As already described, this reflects the different response of infected cells. A rapid hypersensitive response, accompanied by early induction of phytoalexins, arises after infection by incompatible races, whereas the hemibiotrophic behaviour of compatible races of the fungus delays induction of phytoalexin accumulation, which occurs only after the breakdown of the biotrophic phase. The induction of defence-related gene products at the onset of phytoalexin accumulation is coordinated in both incompatible and compatible interactions, especially at the mRNA level (figure 2). The nature of the initial, primary stimulus for expression of phytoalexin biosynthetic genes in the incompatible and compatible interaction is not yet known, although fungal

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elicitors of the types whose effects have been described above are clearly candidates. Another possibility is that some types of elicitor may act by inducing cell death, with resultant release of 'endogenous elicitors' of phytoalexin synthesis from the host. There is now much evidence in the literature to support a role for endogenous factors, most usually pectic fragments of the host cell wall, in elicitation processes, although so far no direct evidence for their involvement has been obtained (for review see Dixon 1986). Elicitor-active fractions have been isolated from bean tissues (Hargreaves & Bailey 1978; Dixon *et al.* 1983*b*) although their chemical nature is not yet clear. The data in figure 2 clearly demonstrate significant induction of enzyme and mRNA activity appearance in areas of tissue adjacent to infected cells which themselves do not yet contain the fungus. This therefore suggests the involvement of intercellularly transmitted host regulatory factors or diffusible fungal components (e.g. cell wall degrading enzymes that may either kill host cells or release elicitor-active wall fragments, or both).

5. Mechanisms underlying the transient appearance of induced gene products

The elicitor-mediated appearance of resistance-related enzymes in bean cell cultures depends upon transient increases in mRNA activities. Initial induction is rapidly followed by a decline in mRNA and enzyme synthetic rate, and often by rapid loss of enzyme activity. A number of studies have provided evidence that 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. Density labelling with ^2H from ^2H_2O has demonstrated that exogenous addition of cinnamic acid may regulate PAL activity levels by a dual mechanism involving inhibition of enzyme synthesis and stimulation of the rate of removal of active enzyme *in vivo* (Shields *et al.* 1982). Northern blotting, solution hybridization–S1 nuclease protection studies and nuclear transcript run-off analyses have shown a rapid cessation of PAL and CHS transcription, and/or removal of hybridizable mRNA, after the addition of cinnamic acid to elicitor-induced cells during the initial period of increased enzyme synthesis; consequently, the mRNA activities encoding PAL, CHS and CHI decline rapidly, resulting in decreased synthesis of the corresponding enzyme subunits (G. P. Bolwell, M. P. Robbins & R. A. Dixon, unpublished). However, addition of cinnamic acid has significantly less effect on the translatable mRNA activity encoding the putative cytochrome P450 that may be involved in cinnamic acid hydroxylation. It is therefore possible that cinnamic acid could act as a cellular modulator for the 'down-regulation' of expression of genes involved in phenylpropanoid metabolism, although the exact specificity of this effect is still not clear. Some effects of cinnamic acid on PAL activity, level and synthesis in elicitor-treated bean cells are summarised in table 3.

In contrast to the rapid loss of PAL activity, extractable activities of elicitor-induced CHS and cinnamic acid 4-hydroxylase are relatively unaffected by exogenous additions of *trans*-cinnamic acid to bean cells (unpublished results), whereas CHI activity can be markedly stimulated by such treatments (Gerrish *et al.* 1985). The effects of cinnamic acid on enzyme removal therefore appear to be specific for PAL. Pulse–chase and immune blotting experiments have indicated that cinnamic acid does not affect the rate of turnover of PAL subunits *in vivo*, but rather mediates irreversible inactivation of the enzyme (Bolwell *et al.* 1986*a*). A non-dialysable factor from cinnamate-treated bean cells, whose formation requires protein and RNA synthesis, stimulates removal of PAL activity from enzyme extracts *in vitro*; this is

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TABLE 3. EFFECTS OF EXOGENOUS APPLICATION OF CINNAMIC ACID ON THE ACTIVITY, SYNTHESIS AND TURNOVER OF PHENYLALANINE AMMONIA-LYASE IN ELICITOR-TREATED BEAN CELL CULTURES

treatment[a]	PAL activity (μkat kg^{-1} protein)	immunodetectable PAL[b] (% maximum elicited value)	rate of PAL synthesis[c] (% maximum elicited value)	PAL polysomal mRNA amount[d] (% maximum elicited value)	PAL turnover[e]
none, zero time	65	27	20	25	—
+elicitor, 4 h	150	41	77	100	100
+elicitor, 8 h	300	91	47	75	74
+elicitor, 8 h (cinnamate added at 4 h)	30	73	25	25	72

[a] Suspension cultured cells of *Phaseolus vulgaris* cv. Canadian Wonder were treated with elicitor (20 μg glucose equivalents ml^{-1}) prepared from the cell walls of *Colletotrichum lindemuthianum* and harvested at the times shown.
[b] Measured by immune blotting.
[c] Measured as [^{35}S]methionine incorporation *in vivo* into immunoprecipitable PAL subunits during a 1 h pulse immediately before the time of harvest.
[d] Measured by solution hybridization of a single stranded ^{32}P-labelled genomic PAL intron:exon boundary probe to polysomal mRNA and analysis of the duplex fragment protected from S1 nuclease digestion.
[e] Expressed as percentage of immunodetectable newly synthesized PAL subunits present at 4 h; cells were labelled with [^{35}S]methionine *in vivo* from 2–4 h after addition of elicitor, and labelled PAL subunits immunoprecipitated from extracts of cells taken during the subsequent chase period with unlabelled methionine.

accompanied by an apparent loss or reduction of the dehydroalanine residue at the PAL active site (as detected by active-site-specific tritiation), although the levels of immunodetectable PAL subunits remain unchanged. Pulse–chase experiments have shown that cinnamic acid-mediated loss of enzyme activity *in vivo* is accompanied by a greater loss of ^{35}S-labelled enzyme subunits precipitated by an immobilized active-site-specific affinity ligand than of subunits precipitated with anti-(PAL) IgG (Bolwell *et al.* 1986*a*), further supporting the active site modification mechanism for cinnamic acid-mediated removal of PAL activity *in vivo*. This effect, in addition to the effects on enzyme synthesis, could provide a rapid, flexible mechanism for down-regulating the flux into the phenylpropanoid pathway in response to an increased cinnamate pool size.

6. CONCLUDING REMARKS

Rapid, induced, resistance responses of plants to microbial pathogens involve the accumulation of a number of gene products, some of which, although related functionally, may be unrelated metabolically (e.g. phytoalexin biosynthetic enzymes and hydroxyproline-rich glycoproteins). A picture is now beginning to emerge in which temporal and spatial patterns of accumulation of defence-related gene transcripts can be seen to underlie the expression of host defence, and this now provides the background for detailed studies on the organization and structure of defence-related genes in terms of their activation by microbial stress. The next few years should see rapid progress in our understanding of the molecular events, resulting from initial pathogen recognition, that determine the localization, timing, extent and coordination of defence gene activation. Furthermore, such studies should also reveal further details of the

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operation of transcriptional and translational control mechanisms during the growth and development of the healthy plant, particularly in relation to those areas of metabolism sharing enzymes (sometimes multigenically encoded) common to those involved in stress-related pathways.

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