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Elicitor rapidly induces chalcone synthase mRNA in *Phaseolus vulgaris* cells at the onset of the phytoalexin defense response

(cDNA cloning/mRNA hybridization/gene expression/isoflavonoid phytoalexins/plant disease resistance)

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ABSTRACT **DNAs complementary to poly(A)$^+$ RNA present in elicitor-treated cells of *Phaseolus vulgaris* L. were inserted into pBR325 and used to transform *Escherichia coli* strain JA221. A clone was identified that contained sequences complementary to mRNA encoding chalcone synthase, a regulatory enzyme of phenylpropanoid biosynthesis, which catalyzes the first reaction of a branch pathway specific to flavonoid and isoflavonoid biosynthesis. Rapid, marked but transient increases in chalcone synthase mRNA in response to elicitor treatment were observed by RNA blot hybridization with ^{32}P-labeled chalcone synthase cDNA sequences. Induction of chalcone synthase mRNA governs the rate of enzyme synthesis throughout the phase of rapid increase in enzyme activity at the onset of accumulation of isoflavonoid-derived phytoalexins. The data are consistent with the hypothesis that elicitor causes a rapid transient stimulation of transcription of chalcone synthase gene(s) as an early event in the expression of the phytoalexin defense response.**

Elucidation of the causal sequence of events leading to expression of defense responses is a key to our understanding of plant disease resistance and future attempts to modify this important trait by gene manipulation. Resistance involves not only static protection but also inducible defense mechanisms, including accumulation of host-synthesized phytoalexin antibiotics, deposition of lignin-like material, accumulation of hydroxyproline-rich glycoproteins, and increases in the activity of certain hydrolytic enzymes (1–3). Infection causes marked changes in the pattern of host protein synthesis (4) and inhibitor studies indicate that disease resistance is an active process dependent on host RNA and protein synthesis (1, 2).

The molecular mechanisms underlying operation of defense responses in biologically stressed plant cells have been most intensively studied in relation to phytoalexin induction (5). Phytoalexin accumulation, which is largely a result of increased synthesis from remote precursors (6, 7), can be induced not only by infection but also by glycan, glycoprotein, and lipid elicitors present in fungal and bacterial cell walls and culture filtrates (8) and by a variety of structurally unrelated, artificial inducers (9). *In vivo* labeling has demonstrated marked but transient increases in the rates of synthesis of phytoalexin biosynthetic enzymes concomitant with the onset of phytoalexin accumulation (10–18). The transient increases in enzyme synthesis reflect increases in the levels of the corresponding mRNA activities (4, 13, 15, 19) that are responsible, in some cases together with changes in the apparent stability of the biosynthetic enzymes *in vivo* (12, 18), for the marked increases in enzyme activity that control expression of the phytoalexin defense response in elicitor-treated cells or infected tissues (5).

A major question in relation to the early molecular events in biologically stressed cells is whether such changes in translatable mRNA activity reflect increased mRNA levels or activation of pre-existing but inactive forms. To address this question we have constructed a cDNA library complementary to poly(A)$^+$ RNA present in elicitor-treated cells of *Phaseolus vulgaris* L. (French bean) and from this library have identified a number of clones containing sequences encoding the enzyme chalcone synthase. Chalcone synthase is a key regulatory enzyme of phenylpropanoid biosynthesis (18–21), catalyzing the first reaction of a branch pathway specific to flavonoid and isoflavonoid biosynthesis (5, 22). Treatment of suspension-cultured cells of *Phaseolus vulgaris* causes marked, transient increases in the rates of synthesis of chalcone synthase and at least three other enzymes of phenylpropanoid biosynthesis (refs. 10–12, 18, and 19; unpublished data), concomitant with the onset of accumulation of phaseollin and structurally related isoflavonoid-derived phytoalexins (5, 11). Increases in the rate of enzyme synthesis can be observed within 20 min of elicitor treatment with maximal rates being obtained 3 hr after elicitation, at which time chalcone synthase mRNA activity accounts for about 1% of total protein synthesis (18, 19).

In the present paper we have used cloned *Phaseolus vulgaris* chalcone synthase cDNA sequences to measure by hybridization the induction kinetics of chalcone synthase mRNA in elicitor-treated cells of *Phaseolus vulgaris*.

MATERIALS AND METHODS

Fungal Cultures and Elicitor Preparation. The source, maintenance, and growth of cultures of *Colletotrichum lindemuthianum* were as described (23). Elicitor was the high molecular weight fraction released by heat treatment of isolated mycelial cell walls (24).

Plant Cell Cultures. Cells of *Phaseolus vulgaris* L. were grown as described, save that cultures were maintained in total darkness (25). All experiments were conducted with 7- to 9-day-old cultures, the medium of which exhibited a conductivity between 2.5 and 2.8 mhos (13).

Enzyme Extraction and Assay. Enzyme was extracted by gently homogenizing 1 g of tissue in 2.0 ml of 0.1 M potassium phosphate (pH 8.0) containing 1.4 mM 2-mercaptoethanol. Cellular debris were removed by centrifugation of the extract for 20 min at 5000 × *g* and chalcone synthase activity was determined with a radioactive assay procedure (21) as described (18). 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.

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Protein Synthesis *in Vivo*. Chalcone synthase was labeled *in vivo* by treatment of elicited cell cultures with [^{35}S]methionine for 30 min prior to harvest as described (18). Chalcone synthase was separated from other labeled proteins in cell extracts by immunoprecipitation with antiserum to *Petroselinum hortense* chalcone synthase followed by NaDodSO$_4$/polyacrylamide gel electrophoresis (18). Chalcone synthase subunits were located by fluorography and [^{35}S]methionine incorporation was determined as described (19). The rate of enzyme synthesis is defined as the extent of incorporation of [^{35}S]methionine into chalcone synthase subunits as a percentage of incorporation into total protein during a 30-min pulse, measured as described (19). Temporal changes in the rate of chalcone synthase synthesis in elicitor-treated cells are expressed relative to the rate of synthesis at maximal induction.

Isolation of RNA. Extraction of RNA from elicitor-treated cells of *Phaseolus vulgaris* has been characterized (19). Polysomal RNA was isolated from cells by a modification (26) of the method of Palmiter (27). Total cellular RNA was isolated from cells homogenized directly in a phenol/0.1 M Tris·HCl, pH 9.0, emulsion as described by Haffner *et al.* (28). Further purification of the phenol-extracted total RNA was identical to that used for polysomal RNA (27).

Protein Synthesis *in Vitro*. Isolated polysomal RNA or total cellular RNA was translated *in vitro* in the presence of [^{35}S]methionine by using an mRNA-dependent rabbit reticulocyte lysate translation system (29) and incorporation of [^{35}S]methionine into total protein was measured as described (19). Chalcone synthase was separated from other translation products by indirect immunoprecipitation with antiserum to *Petroselinum hortense* chalcone synthase and protein-A Sepharose followed by NaDodSO$_4$ polyacrylamide gel electrophoresis (19). Chalcone synthase mRNA activity is defined as the incorporation of [^{35}S]methionine into immunoprecipitable chalcone synthase subunits as a percentage of incorporation into total protein. Temporal changes in chalcone synthase mRNA activity in elicitor-treated cells are expressed relative to the mRNA activity at maximal induction.

Cloning Procedure. Poly(A)$^+$ RNA was isolated by two cycles of oligo(dT)-cellulose affinity chromatography of polysomal RNA prepared from cells of *Phaseolus vulgaris* 3 hr after treatment with elicitor (30). Blunt-ended, double-stranded cDNA was synthesized as described (31). One-half microgram of the product was treated with *Eco*RI methylase and *S*-adenosylmethionine by using conditions recommended by the enzyme supplier (Bethesda Research Laboratories). After phenol extraction and ethanol precipitation of the cDNA, kinase-treated, synthetic oligonucleotide linkers containing the *Eco*RI recognition sequence were ligated to the cDNA (32). The linkers were cleaved from the cDNA by incubation with 500 units of *Eco*RI (Boehringer Mannheim) for 8 hr at 37°C. To separate completely the cDNA from the cleaved linkers, the sample (50 μl) was loaded into the 3 × 50 mm well of a 3% acrylamide/0.15% bisacrylamide gel that was run at 50 V until the leading edge of the bromophenol blue had migrated 6 mm into the gel. The top 3 mm of the lane was cut out and the DNA was electroeluted from the gel slice. To prepare the vector, 5 μg of pBR325 was digested with *Eco*RI. The linear form was purified by agarose gel electrophoresis, electroelution of the excised band, phenol extraction of the eluate, and ethanol precipitation. The vector was treated with 1 unit of calf intestine alkaline phosphatase (Boehringer Mannheim) for 1 hr at 37°C, after which nitriloacetate (pH 8.0) was added to a final concentration of 10 mM and the sample was incubated at 65°C for 1 hr. After phenol extraction and ethanol precipitation the vector was combined with the cDNA and incubated with 2 units of T4 DNA ligase (Bethesda Research Laboratories) at 12°C for 16 hr. The ligated products were transformed (33) into a derivative of the *Escherichia coli* strain JA221 (*lpp hasR$^-$ hasM$^+$ trpE leu lacY recA1*) and transformants were selected by plating on L plates containing 15 μg of tetracycline per ml.

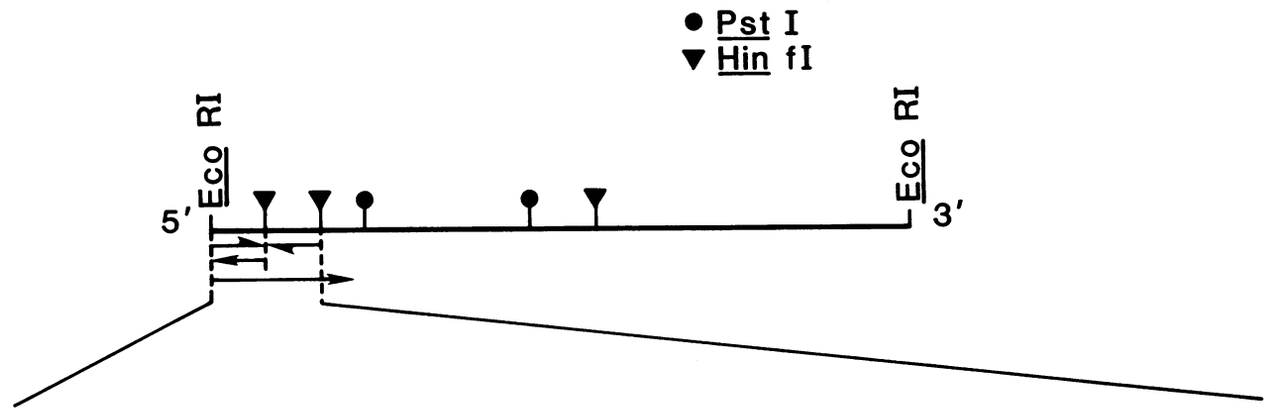
Screening and Identification. Colony hybridization was performed as described by Grunstein and Hogness (34). The probe was a cDNA sequence complementary to an irradiation-induced chalcone synthase mRNA associated with flavonoid pigment formation in *Petroselinum hortense* (35). The probe was cleaved from pLF15 (35) by digestion with *Pst* I and labeled by nick-translation with DNA polymerase I and [α-^{32}P]dNTPs (New England Nuclear). Hybridization was carried out at 42°C, in a buffer containing 30% formamide, 0.9 M NaCl, 0.09 M sodium citrate (pH 7.0), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 5 mM EDTA, 0.1% NaDodSO$_4$, and 100 μg of calf thymus DNA per ml. Hybridization was for 48 hr after a preincubation of the filters for 12 hr in the absence of probe. Plasmid DNA was prepared from selected positive clones (36), digested with *Eco*RI, separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with nick-translated pLF15 (37). Hybridization conditions were as described above. Nucleotide sequences were determined by the method of Maxam and Gilbert (38).

RNA Blot Hybridization. RNA (8 μg) was denatured with glyoxal, separated by electrophoresis on a 1.2% agarose gel in 10 mM phosphate buffer (pH 7.0) (39), blotted onto nitrocellulose (40), and hybridized with a probe prepared by digesting pCHS1 with *Eco*RI, followed by nick-translation and agarose gel electrophoresis of the labeled DNA. Hybridization conditions were as described above except that 50% formamide, 0.45 M NaCl, and 0.045 M sodium citrate (pH 7.0) were used.

Hybrid Selection of Chalcone Synthase mRNA. Chalcone synthase mRNA was selected by hybridization to filter-bound cDNA as described (31). pCHS1 was linearized with *Hin*dIII, extracted with phenol, and precipitated with ethanol. Ten micrograms was adsorbed to each of two nitrocellulose squares (3 × 3 mm). The duplicate filters were incubated in a 20-μl hybridization mix (31) with 10 μg of poly(A)$^+$ RNA from induced cells harvested 3 hr after elicitor treatment and from uninduced control cells, respectively. The eluate from each filter was precipitated with ethanol and the RNA was resuspended in 10 μl of water. Four microliters of each sample was translated *in vitro* and compared to the translation products of 4 μg of poly(A)$^+$ RNA from induced and uninduced cells by NaDodSO$_4$/polyacrylamide gel electrophoresis as described above.

RESULTS

From about 20,000 colonies in a library containing sequences complementary to mRNA from elicitor-treated cells of *Phaseolus vulgaris*, 48 were found to hybridize *in situ* with *Petroselinum hortense* chalcone synthase cDNA sequences. Twenty of these were chosen for blot-hybridization analysis (37) of *Eco*RI-digested plasmid DNA. Six contained plasmids with inserts larger than 1 kilobase. The plasmid pCHS1 contains an insert of about 1.4 kilobases at the *Eco*RI site of the cloning vehicle. The isolated insert hybridized to *Petroselinum hortense* chalcone synthase cDNA sequences in Southern blot analysis and its identity was confirmed by comparison of a partial DNA sequence with that of *Petroselinum hortense* chalcone synthase cDNA (41) (Fig. 1). In this region of 226 base pairs, the *Phaseolus vulgaris* and *Petroselinum hortense* DNA sequences were about 67% homologous. There were termination codons in two of the three reading frames of the 226-base-pair *Phaseolus vulgaris* sequence and in the open reading frame most of the nucleotide substitutions between this sequence and the *Petroselinum hortense* sequence occurred in the third base position of the

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          1                                                                                                    112
A:        AATTCCGAGATCCGCCAGGCTCAAAGGGCAGAAGGCCCAGCAACCATCCTTGCCATAGGAACTGCAACCCCATCAAACTGTGTTGATCAAAGCACATATCCTGATTACTACT
          ** *** * *    ** ** **  **** ** ** *  ** ** ** ** ** ***** ** ** ** ********  * **       ****************
B:          GAAATCAGGAACAGGCAGAGAGCCCAAGGTCCTGCCAATATACTAGCTATTGGCACTGCCACTCCTTCCAACTGTGTCTACCAGGCTGATTATCCTGATTACTACT
          68                                                                                                   173

                                                                                                               232
TCAGAATCACAAACAGTGAGCACATGACCGACCTCAAAGAGAAGTTCCAGCGCATGTGTGACAAGTCGATGATAAAGAAGAGATATATGCACCTGGACGAGGAGATACTGAAGGAGATTC
*  * ** ** ***   ** ********** ******   ** *** **** ******** ** ** ***** *  *** * ** *****  *    *****    ***** ** *  *
TTCGTATTACCAACTCCGAACACATGACCGATCTCAAACTCAAATTCAAGCGAATGTGTGAAAAATCAATGATCAGAAAGCGTTACATGCATATAACAGAGGAATATCTGAAAGAAAACC
                                                                                                               293
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FIG. 1. Restriction map of the *Phaseolus vulgaris* chalcone synthase cDNA sequence (1.4 kilobase) present in plasmid pCHS1. The designations 5′ and 3′ correspond to the polarity of the corresponding mRNA. The arrows show the direction in which the indicated restriction fragments were sequenced. The DNA sequence of one end of pCHS1 (A) is compared with part of the sequence of *Petroselinum hortense* chalcone synthase cDNA (B) from pLF15 (41). Note that the first six bases of the pCHS1 sequence correspond to the *Eco*RI linker sequence. The numbers below the pLF15 sequence correspond to the published coordinates (41).

respective codons, resulting in codons that were synonymous in each sequence. The predicted peptides encoded by the *Petroselinum hortense* sequence and the corresponding reading frame of the *Phaseolus vulgaris* sequence were 79% homologous. Preliminary nucleotide sequence analysis of other regions of the pCHS1 cDNA insert, including the 3′ end region, showed that no other cDNA species were fused to chalcone synthase sequences during the cloning procedures. Detailed information on the structure and sequence of this and other *Phaseolus vulgaris* chalcone synthase cDNA sequences will be published elsewhere.

Hybrid selection/*in vitro* translation confirmed that the mRNA species that hybridize with pCHS1 encode the polypeptides precipitated by antiserum to chalcone synthase (Fig. 2). RNA from control cells selected by hybridization to filter-bound pCHS1 showed almost no mRNA activity (lanes d and f). In contrast, the selected RNA from elicitor-treated cells showed strong mRNA activity for a small number of

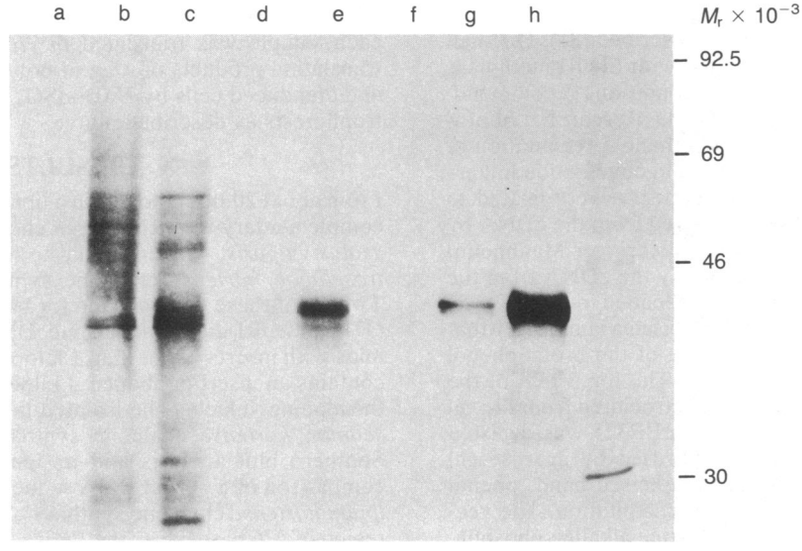

FIG. 2. *In vitro* translation of induced and uninduced poly(A)$^+$ RNA and hybrid-selected chalcone synthase mRNA. Substrates for the *in vitro* translations were no exogenous RNA control (lane a), poly(A)$^+$ RNA from untreated cells (lane b), poly(A)$^+$ RNA from elicitor-treated cells (lanes c and g), hybrid-selected RNA from untreated cells (lanes d and f), and hybrid-selected RNA from elicitor-treated cells (lanes e and h). For lanes a–e, 2.5 μl of the respective ^{35}S-labeled polypeptide products was mixed with sample buffer and directly loaded onto the gel. For lanes f–h, 20 μl of the respective ^{35}S-labeled polypeptide products was treated with antiserum to chalcone synthase; the immunoprecipitated material was resuspended in sample buffer and applied to the gel.

polypeptide species (lane e) that were immunoprecipitated by antiserum to chalcone synthase (lane h). Most of the immunoprecipitated chalcone synthase translated from hybrid-selected mRNA (lane h) comigrated with chalcone synthase translated from poly(A)$^+$ RNA (lane g). However, a small amount of lower molecular weight material from hybrid-selected RNA probably represented translation products of slightly degraded chalcone synthase mRNA.

The chalcone synthase cDNA insert generated by restriction of pCHS1 with *Eco*RI was separated from the pBR325 cloning vehicle and used to measure the kinetics of chalcone synthase mRNA induction in elicitor-treated cells of *Phaseolus vulgaris*. Total and polysomal RNA was isolated from cells at various times after elicitation. ^{32}P-labeled *Phaseolus vulgaris* chalcone synthase cDNA specifically hybridized with an RNA species of about 1.5 kilobases.

Chalcone synthase mRNA was almost completely absent from control cells but was rapidly induced following elicitor treatment (Fig. 3). Increases in chalcone synthase mRNA could be observed 30 min after elicitation and maximal induction occurred about 3 hr after elicitor treatment. Subsequently, the mRNA rapidly decayed to relatively low levels.

There is a weak induction of enzyme activity and enzyme synthesis in the absence of elicitor, arising from experimental manipulation of the cell cultures at the start of the time course (Fig. 4*A* and ref. 19). Similarly, RNA transfer blot analysis showed a slight induction of hybridizable chalcone synthase mRNA in cells receiving buffer rather than elicitor. The chalcone synthase RNA level in these cells at 3 hr was <1% of the level in equivalent elicitor-treated cells (data not shown).

Induction and decay of chalcone synthase mRNA in total and polysomal RNA fractions exhibited very similar time courses (Fig. 4). Furthermore, there was a close correlation between the kinetics of induction of hybridizable mRNA, translatable mRNA activity, and chalcone synthase synthesis *in vivo* (Fig. 4). Maximal induction coincided with the period of most rapid increase in chalcone synthase enzyme activity.

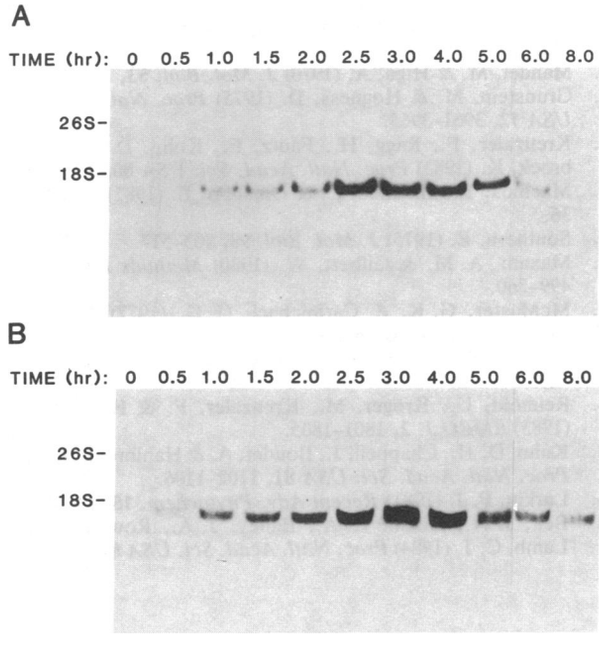

FIG. 3. Induction of chalcone synthase mRNA in elicitor-treated cells of *Phaseolus vulgaris*. Polysomal RNA (*A*) and total cellular RNA (*B*) were isolated from cells at various times after elicitor treatment and fractionated by electrophoresis in agarose gels. After transfer to nitrocellulose, chalcone synthase mRNA was detected by hybridization with ^{32}P-labeled cDNA from pCHS1 followed by autoradiography.

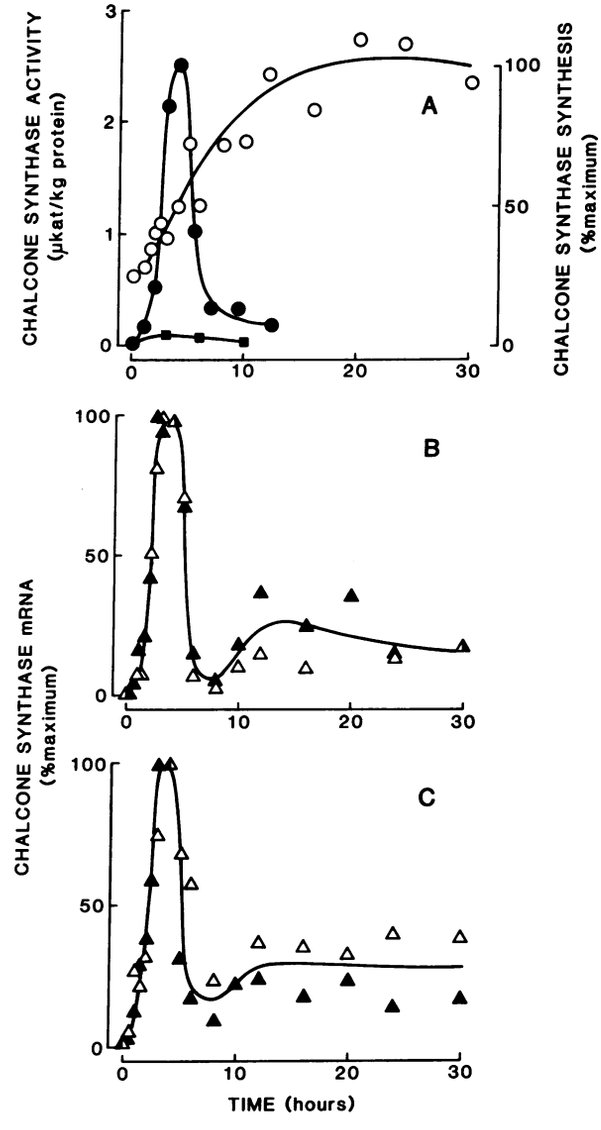

FIG. 4. Induction kinetics of chalcone synthase in elicitor-treated cells of *Phaseolus vulgaris*. (*A*) Enzyme activity (○) in treated cells and rate of enzyme synthesis *in vivo* in treated cells (●) and mock-treated cells (■); (*B*) hybridizable mRNA (▲) and translatable mRNA activity (△) in the polysomal RNA fraction; (*C*) hybridizable mRNA (▲) and translatable mRNA activity (△) in the total cellular RNA fraction.

DISCUSSION

Previous studies have established the quantitative importance of induction of enzyme synthesis in the regulation of chalcone synthase levels during expression of the phytoalexin defense response in *Phaseolus vulgaris* (18, 19). The present data show that changes in the amount of chalcone synthase mRNA underlie elicitor-stimulated increases in mRNA activity and enzyme synthesis. Induction of mRNA was observed in both total cellular RNA and polysomal RNA fractions, eliminating the possibility that mRNA activity was not detected in control, uninduced cells because of sequestration of RNA from polysomes. The close correspondence between the induction kinetics of translatable mRNA activity and amount of hybridizable mRNA in both total cellular RNA and polysomal RNA fractions indicates that the transient induction of chalcone synthase mRNA is the major fac-

tor governing the rate of enzyme synthesis throughout the phase of rapid increase in enzyme activity. Transient induction of the mRNA encoding 4-coumarate:CoA ligase, an enzyme of general phenylpropanoid metabolism, concomitant with increased enzyme synthesis associated with accumulation of furanocoumarin phytoalexins has recently been observed in elicitor-treated cells of *Petroselinum hortense* (42).

The rapidity of chalcone synthase mRNA induction implies that this is not a nonspecific, secondary effect of elicitor treatment but rather a specific response that is an early component in the causally related sequence of events between elicitor binding to a putative plant cell receptor (43) and accumulation of phytoalexins. Furthermore, the rapid induction of mRNA from very low basal levels strongly suggests that elicitor initially causes a transient increase in the transcription of chalcone synthase gene(s), although control over mRNA processing or degradation are alternative possibilities. Post-transcriptional control mechanisms may become important in the later stages of the response especially in relation to the subsequent rapid decay in chalcone synthase mRNA. Delineation of the roles of transcription, RNA processing, and RNA turnover in determining the timing, magnitude, and duration of chalcone synthase mRNA induction in elicitor-treated cells will require the use of RNA-labeling procedures.

The highly coordinated increases in synthesis of a number of enzymes of isoflavonoid phytoalexin biosynthesis in both elicitor-treated cells (refs. 18 and 19; unpublished data) and infected tissue (unpublished data; ref. 44) of *Phaseolus vulgaris* argues strongly that a similar mechanism of induction operates for many if not all enzymes in the pathway. Hence, the picture that begins to emerge from these studies is of rapid and extensive, selective changes in the pattern of gene expression underlying the induction of phytoalexin accumulation and possibly other defense responses in biologically stressed plant cells.

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