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Chalcone isomerase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection

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The environmentally regulated synthesis of phenylpropanoid natural products was studied by examining the expression of the gene encoding chalcone isomerase (CHI). This enzyme catalyzes a step common to the synthesis of flavonoid pigments and isoflavonoid phytoalexins. A λgt11 library was constructed using mRNA from cell cultures of bean (*Phaseolus vulgaris* L.) treated with fungal elicitor. Two positive clones were obtained by screening 10^5 recombinants with an antiserum to purified bean CHI. The identity of the cloned sequences was confirmed by hybrid-select translation and the production of antigenic polypeptides from transcripts synthesized *in vitro*. Addition of elicitor to cell cultures resulted in the rapid accumulation of CHI mRNA, with maximum levels achieved 3–4 h after elicitation. CHI mRNA also accumulated during the natural infection of hypocotyls with the fungal pathogen *Colletotrichum lindemuthianum*, and in mechanically wounded hypocotyls. The kinetics of accumulation of CHI mRNA in response to these environmental signals were strikingly similar to those of mRNAs encoding two other phenylpropanoid pathway enzymes, phenylalanine ammonia-lyase and chalcone synthase. In contrast to the multi-gene families encoding these two enzymes, chalcone isomerase is encoded by a single gene which is regulated by several environmental stimuli.

Key words: chalcone isomerase/chalcone synthase/phenylalanine ammonia-lyase/*Phaseolus vulgaris* L./*Colletotrichum lindemuthianum*/infection/wounding

Introduction

Plants exhibit a number of adaptive and protective responses to environmental stress. Of particular significance is the production of diverse phenylpropanoid natural products elaborated from phenylalanine including the cell wall polymer lignin and flavonoid pigments, as well as isoflavonoid and coumarin phytoalexin antibiotics (Hahlbrock and Grisebach, 1979; Dixon *et al.*, 1983a). The synthesis of these products by specific branch pathways which diverge from the central phenylpropanoid pathway is selectively modulated during development and by environmental stimuli. For example, u.v. and white light stimulate the production of protective u.v.-absorbing flavonoids (Hahlbrock and Wellmann, 1970; Hahlbrock *et al.*, 1976) whereas infection induces phytoalexin synthesis (Rathmell and Bendall, 1971; Whitehead *et al.*, 1982). We are interested in understanding the molecular mechanisms leading to these protective responses.

To investigate mechanisms of defense against infection, we are studying the interaction of bean (*Phaseolus vulgaris* L.) with the fungal pathogen *Colletotrichum lindemuthianum*, the causal agent of anthracnose disease (Bailey and Deverall, 1971). Previous studies have shown that application of a fungal cell wall preparation (elicitor) to bean cell suspension cultures causes rapid increases in the activities of at least five enzymes of phenylpropanoid biosynthesis, concomitant with the onset of phytoalexin accumulation (Dixon and Bendall, 1978; Dixon and Lamb, 1979). These increases are correlated with increased rates of synthesis *in vivo* of three enzymes, phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and chalcone isomerase (CHI) (Dixon and Lamb, 1979; Cramer *et al.*, 1985a). Furthermore, the mRNAs encoding these enzymes rapidly accumulated after elicitor treatment as measured by *in vitro* translation and immunoprecipitation (Cramer *et al.*, 1985a).

CHI catalyzes the stereospecific conversion of chalcones to their corresponding flavanones, which is the second reaction of a branch pathway of phenylpropanoid biosynthesis specific for the production of flavonoid pigments and isoflavonoid phytoalexins (Figure 1; Dixon *et al.*, 1983a). Several observations suggest that CHI may be a regulatory enzyme in the synthesis of both classes of compounds. First, various environmental and developmental stimuli result in increased enzyme synthesis and activity (Wiermann, 1972; Forkmann and Kuhn, 1979; Dixon *et al.*, 1983b; Cramer *et al.*, 1985a). Second, the enzyme is strongly inhibited by two flavonols, quercetin and kaempferol, and the isoflavonoids, kievitone and coumestrol (Dixon *et al.*, 1982). Third, induction of CHI enzyme activity by the first intermediate in the phenylpropanoid pathway, cinnamic acid, has also been reported (Gerrish *et al.*, 1985). The enzyme has been purified to homogeneity from bean cell suspension cultures and monospecific antiserum characterized (Dixon *et al.*, 1982; Robbins and Dixon, 1984). Bean CHI is a monomeric protein whose mol. wt is 27 kd.

In the present paper, we describe the identification of CHI cDNA clones by antibody screening of a λgt11 library constructed from mRNA isolated from elicitor-treated bean cells. We demonstrate that there is a single CHI gene within the bean genome and examine its expression in elicitor-treated cells and wounded or infected hypocotyls. These stimuli result in marked accumulation of CHI mRNA with very similar kinetics to those previously observed for mRNAs encoding PAL and CHS (Ryder *et al.*, 1984; Cramer *et al.*, 1985a; Edwards *et al.*, 1985; Bell *et al.*, 1986). These data strongly suggest coordinate regulation of the genes encoding these phenylpropanoid pathway enzymes.

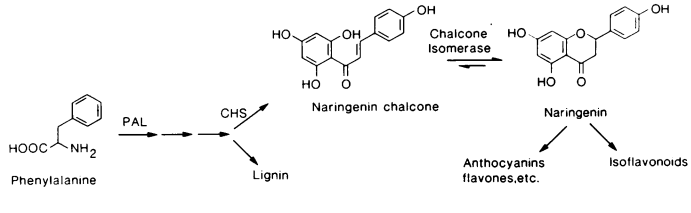

Fig. 1. Metabolic role of CHI in phenylpropanoid biosynthesis.

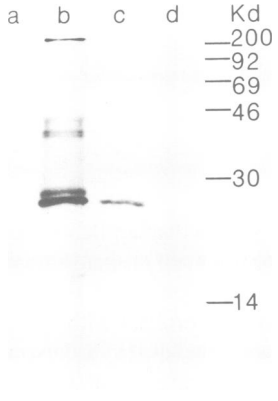

Fig. 2. Immunoprecipitation with CHI antiserum of *in vitro* translation products from pCHI1 hybrid-selected mRNA. RNA samples were translated in a reticulocyte lysate in the presence of [^{35}S]methionine. The translation products were incubated with CHI antiserum, then immunocomplexes were precipitated with protein A-Sepharose. Immunoprecipitates of translation products were separated on a 15% polyacrylamide gel. No added RNA control (**lane a**); poly(A)$^+$ RNA (1 μg) from elicitor-treated cell cultures (**lane b**); poly(A)$^+$ RNA from elicitor-treated cells that had hybridized to filter-bound pCHI1 (**lane c**) and filter-bound pSP65 (**lane d**).

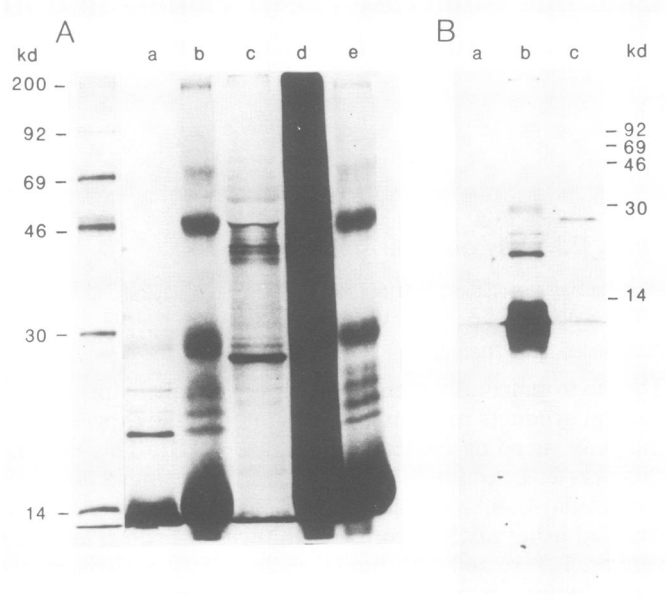

Fig. 3. CHI antiserum immunoprecipitates polypeptides encoded by the pCHI1 cDNA clone. pCHI1, which contains the insert in the sense orientation, was transcribed by SP6 polymerase in the presence of the cap analog G(5′)ppp(5′)G. The *in vitro* transcripts and poly(A)$^+$ RNA from elicitor-treated cell cultures (1 μg of each) were translated and aliquots of the products immunoprecipitated as described in the legend to Figure 2.
(A) The reaction products were separated on a 10% polyacrylamide gel. Immunoprecipitated (**lane a**) and total (**lane b**) pCHI1-derived RNA translation products; immunoprecipitated (**lane c**) and total (**lane d**) poly(A)$^+$ RNA translation products; control without added RNA (**lane e**). The far left lane contains mol. wt standards as indicated.
(B) Immunoprecipitated reaction products were separated on a 20% polyacrylamide gel. Control without added RNA (**lane a**); pCHI1-derived RNA (**lane b**) and poly(A)$^+$ RNA from elicitor-treated cells (**lane c**).

Results

Identification of CHI cDNA clones

A cDNA library was constructed from mRNA isolated from suspension cultured bean cells treated wtih elicitor for 3.5 h. The level of the CHI mRNA is maximal at this time as determined by *in vitro* translation and immunopreciptation (Cramer *et al.*, 1985a). The use of the λgt11 expression vector (Young and Davis, 1983a,b) allowed us to screen for CHI cDNA clones with an antiserum that had been raised against bean CHI purified to homogeneity (Robbins and Dixon, 1984). Two positive clones were identified from a screen of 10^5 recombinants. The cDNA inserts were excised with *Eco*RI and cloned into pSP65. The largest insert (865 bp) was isolated from the plasmid pCHI1, ^{32}P-labeled by nick-translation and used to screen the library for additional CHI cDNA clones. Thirteen additional clones were identified and their cDNA inserts were found to be slightly smaller than the pCHI1 insert which is 86% of the full-length mRNA size (see below).

To demonstrate that the nucleotide sequence of pCHI1 is complimentary to that of CHI mRNA, hybrid-selection of RNA isolated from elicitor-treated cell cultures was carred out. Poly(A)$^+$ RNA was hybridized to filters with either immobilized pCHI1 or pSP65 vector DNA, then bound RNA was eluted and translated *in vitro* in a reticulocyte lysate system. For comparison, total poly(A)$^+$ RNA was also translated then all reactions were incubated with the CHI antiserum. The major immunoprecipitated species (Figure 2, lane b) had a mol. wt of 27 kd, which is the size of the CHI polypeptide (Robbins and Dixon, 1984). The filter bearing pCHI1 selected an mRNA encoding a polypeptide which was immunoprecipitated by the CHI antiserum (lane c) and which co-migrated with the authentic CHI polypeptide (lane b). In contrast, RNA similarly processed with bound pSP65 DNA failed to produce polypeptides which were immunoprecipitable (lane d). Therefore, the plasmid pCHI1 specifically hybridized to an mRNA that encoded a polypeptide which was both recognized by the antibody and had the appropriate mol. wt.

To confirm further the identity of the cDNA clone, we investigated whether polypeptide(s) encoded by the pCHI1 insert were recognized by the CHI antiserum. As the insert was located downstream of the bacteriophage SP6 promoter, capped RNA transcripts with the same polarity as CHI mRNA were generated by *in vitro* transcription with SP6 RNA polymerase. Analysis of the transcripts by agarose gel electrophoresis revealed a single species of the same size as the insert. Figure 3A shows that translation of this RNA *in vitro* generated a set of products (lane b) which by gel electrophoresis could not be distinguished from the translation products of endogenous RNA in the translation system (lane e). However, the immunoprecipitate of the translation products of the pCHI1 transcript contained 3−4 polypeptides, including a major polypeptide which migrated near the ion front (lane a). The authentic 27-kd CHI polypeptide was resolved in the immunoprecipitate (lane c) of translation products from poly(A)$^+$ RNA from elicitor-treated cell cultures (lane d). For improved resolution, immunoprecitated products were separated on a higher percentage (20%) polyacrylamide gel (Figure 3B). The pCHI1 transcript produced a highly abundant 11.4-kd polypeptide as well as several higher mol. wt species which were all precipitated by the CHI antiserum (lane b). These polypeptides were not present in the control without added RNA (lane a) and therefore were encoded by the exogenous pCHI1 transcript. Thus, transcripts from the less than full length cDNA

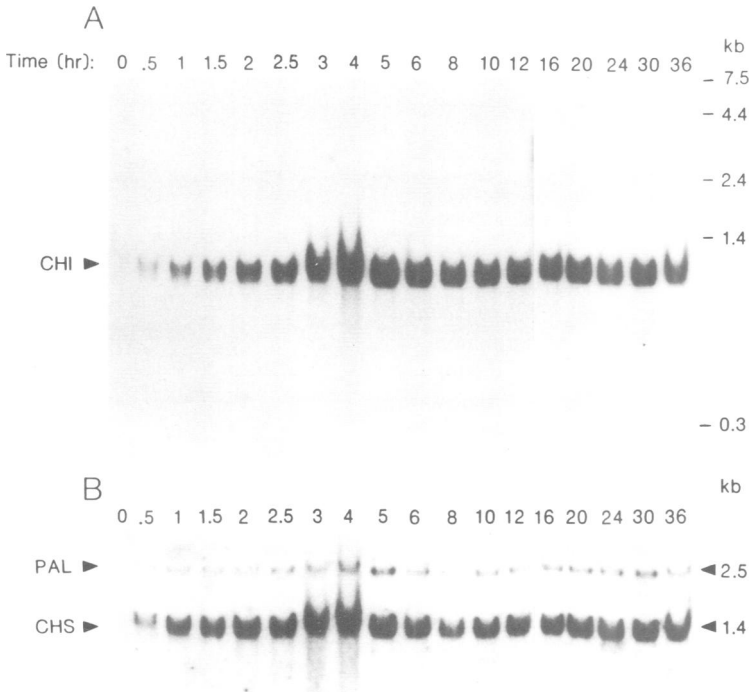

Fig. 4. (**A**)Time course of CHI mRNA accumulation in suspension cultured cells in response to elicitor. Total RNA was isolated at various times (h) after elicitor addition, size separated on an agarose gel (5 μg/lane) and blotted onto nitrocellulose. The blot was hybridized with labeled pCHI1 insert. (**B**) The same blot was washed and re-hybridized with labeled PAL and CHS cDNA inserts.

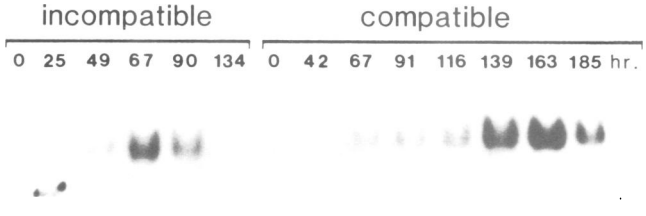

Fig. 5. Time course of CHI mRNA accumulation in hypocotyls of bean cultivar Kievitsboon Koekoek infected with physiological races of *C. lindemuthianum*. Hypocotyl segments were inoculated at $t = 0$ with spores of either the incompatible race β or the compatible race γ. Total RNA was isolated at various times (h) from tissue directly beneath the site of spore inoculation. The RNAs were analyzed as described in the legend to Figure 4.

clone directed the synthesis of polypeptides which were truncated compared to the authentic CHI polypeptide (lane c) but nonetheless were antigenic. These data together with the data from the hybrid-selection experiment established that pCHI1 is complementary to CHI mRNA sequences.

Elicitor induces CHI mRNA accumulation

It has previously been shown that translatable CHI mRNA activity increases in cell cultures which have been treated with fungal elicitor (Cramer *et al.*, 1985a). To determine whether this is due to the *de novo* accumulation of CHI mRNA sequences, blot hybridization of RNA extracted from cell cultures at various intervals after addition of elicitor was performed. ^{32}P-labeled pCHI1 cDNA insert hybridized to a single RNA species of 1.0 kb size, which accumulated within 0.5 h of elicitor treatment (Figure 4A). This mRNA is sufficiently large to encode the 27-kd CHI polypeptide. Maximum mRNA levels occurred at 3–4 h after elicitor treatment. Subsequently, the amount of hybridizable CHI mRNA decreased to stable, approximately half-maximal levels.

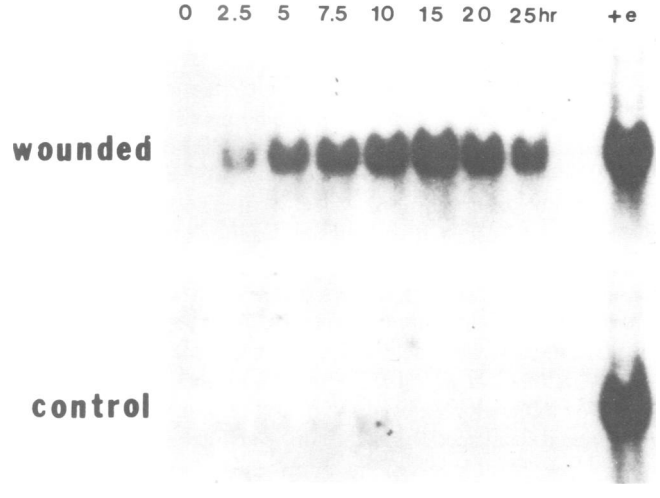

Fig. 6. Time course of CHI mRNA accumulation in excised segments of bean hypocotyls. Etiolated hypocotyls were cut into 10 mm segments and incubated in the dark. At the indicated times (h), aliquots of wounded tissue and control tissue from intact plants were harvested. RNA was analyzed as described in the legend to Figure 4. The lane labelled +e contains RNA from cell cultures treated with elicitor for 3.5 h as a reference.

The effect of elicitor on CHI mRNA levels measured by blot hybridization closely paralleled the previously observed changes in translatable CHI mRNA activity (Cramer *et al.*, 1985a). To compare directly elicitor effects on CHI mRNA accumulation with the accumulation of PAL and CHS mRNAs, the CHI probe was washed from the blot and the blot was rehybridized with PAL and CHS probes. As seen in Figure 4B, the kinetics for accumulation of PAL and CHS mRNA were very similar to those observed for CHI mRNA.

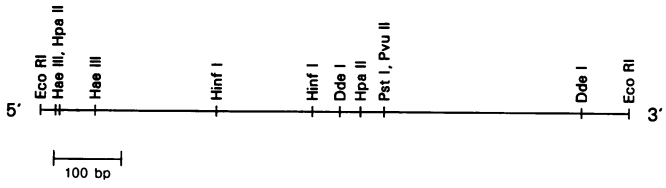

Fig. 7. Restriction map of pCHI1. The map was obtained by end-labeling and partial digestion and confirmed by experiments with complete digestion.

Induction of CHI mRNA by infection and wounding

Infection of bean with different races of *Colletotrichum lindemuthianum* results in different kinetics of induction of phenylpropanoid pathway enzymes and phytoalexin accumulation (Bell *et al.*, 1984, 1986; Cramer *et al.*, 1985a). Application of race β spores to hypocotyls of the cultivar Kievitsboon Koekoek results in localized, rapid accumulation of phytoalexins and hypersensitive death of plant cells within the immediate vicinity. In such an incompatible interaction, fungal growth is restricted to the host cells which are initially penetrated. In contrast, in a compatible interaction with race γ, an extensive mycelium develops. Phytoalexin synthesis is delayed by several days in comparison to the incompatible interaction and occurs only after anthracnose lesions have begun to develop (Bell *et al.*, 1984; O'Connell *et al.*, 1985).

Changes in CHI mRNA levels were examined in bean hypocotyls following infection with spores of race β or race γ (Figure 5). In both cases, germination and formation of an infection peg occurred over an average period of 36 h. Inoculation of hypocotyl segments with the incompatible race β resulted in the rapid accumulation of CHI mRNA in tissue immediately beneath the site of spore inoculation. CHI mRNA became detectable by 49 h after spore inoculation, reached peak levels at 67 h and then declined to undetectable levels. In contrast, inoculation of hypocotyls with race γ resulted in delayed accumulation of CHI mRNA. In this case, the mRNA first became detectable at 67 h and maximal levels were not observed until ~163 h after spore inoculation. Control hypocotyls treated with sterile water exhibited low, basal CHI mRNA levels (data not shown). These data are in agreement with previous measurements of CHI mRNA levels by *in vitro* translation and immunoprecipitation (Cramer *et al.*, 1985a).

As phytoalexins also accumulate in mechanically wounded tissue (Whitehead *et al.*, 1982), we determined whether CHI mRNA was induced in excised hypocotyl segments. Hypocotyls from etiolated bean seedlings were utilized since this tissue contains very low, basal levels of phenylpropanoid pathway enzymes and mRNAs (Bell *et al.*, 1986). As seen in Figure 6, the wounded hypocotyl segments accumulated CHI mRNA within 2.5 h following excision and maximum levels were obtained after 15 h. In contrast, the level of CHI mRNA remained very low in control hypocotyls isolated from intact plants over the same period. Since the wounded and control RNAs were resolved on two separate gels, equivalent amounts of RNA from elicitor-treated cell cultures were included as controls for blotting and hybridization efficiencies. These data demonstrate that CHI mRNA is strongly induced during infection and in response to wounding. Rehybridization of the blots with PAL and CHS probes demonstrated that PAL and CHS mRNAs accumulate with similar kinetics to those observed for CHI mRNA (data not shown; Bell *et al.*, 1986).

Evidence for a single CHI gene

Restriction site analysis of independently isolated CHI cDNA clones was conducted in order to check for restriction site heterogeneity which might reflect the existence of multiple CHI genes. Eight clones, of which pCHI1 was the largest, possessed identical restriction maps (Figure 7). We suggest from these data that a single CHI transcript accumulates in response to elicitor. This interpretation is consistent with the observation that only one CHI polypeptide could be detected by two-dimensional gel electrophoretic analysis of immunoprecipitated CHI synthesized *in vitro* by translation of polysomal RNA isolated from elicitor-induced cells (Figure 8). Therefore, it appears that a single CHI gene is expressed in response to elicitor.

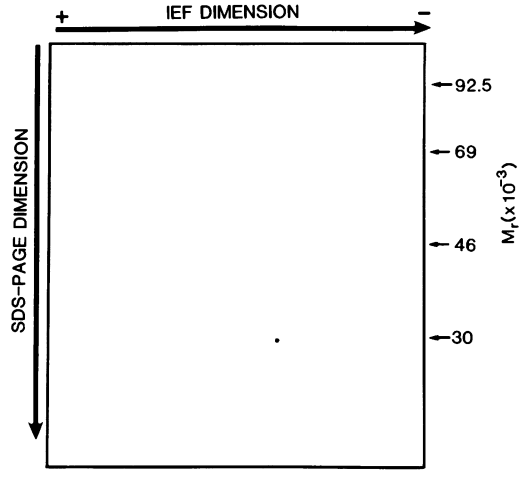

Fig. 8. Two-dimensional gel electrophoresis of polypeptides synthesized by *in vitro* translation and precipitated with CHI antiserum. The RNA used to direct translation was polysomal RNA isolated from cell cultures 3 h after elicitor treatment.

To determine whether more than one CHI gene could be detected within the bean genome, labeled pCHI1 was hybridized to genomic DNA digested with several enzymes which do not cleave within the cDNA insert. Hybridization to DNA isolated from either cell cultures (Figure 9A) or leaves (Figure 9B) was compared. Both DNA samples contained single hybridizing bands of identical sizes in the *Bam*HI, *Eco*RI and *Sal*I digests. The larger hybridizing *Sal*I fragment (Figure 9A) and *Eco*RI fragment (Figure 9B) were due to incomplete digestion and were not reproducibly observed (data not shown). The *Sal*I digest was complete in (B) and the *Eco*RI digest was complete in (A). *Xba*I and *Cla*I digests also contained single hybridizing fragments. From these results, we conclude that there is a single CHI gene in the bean genome and, furthermore, that this gene has not rearranged or duplicated in the cell cultures from which the cDNA clone was prepared. Only the *Hin*dIII digests consistently showed two hybridizing bands and this might reflect the presence of an intron containing a *Hin*dIII site. In contrast to these findings for CHI, multiple PAL and CHS genes have been shown by restriction site and sequence analysis as well as two-dimensional gel electrophoresis of immunoprecipitated products synthesized *in vitro* by translation of isolated RNA (Bell *et al.*, 1986; Ryder *et al.*, submitted; Cramer and Lamb, unpublished).

Discussion

We have identified cDNA clones of the bean CHI gene. This study utilized these clones as probes to show that elicitor treatment, infection and wounding induce the rapid accumulation of CHI mRNA coordinately with mRNAs encoding PAL and CHS.

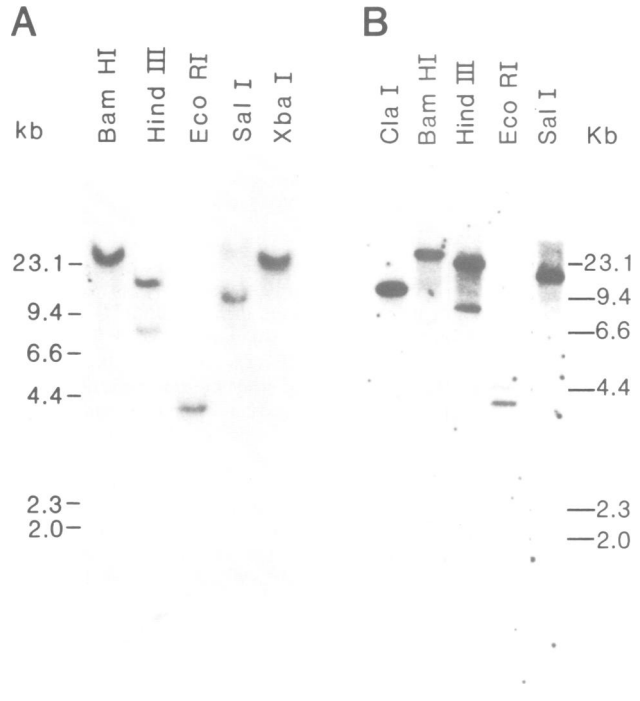

Fig. 9. Genomic blot analysis. Genomic DNA samples (3 μg) from bean cultivar Canadian Wonder cell cultures (**A**) and leaves (**B**) were digested with the indicated restriction enzymes and the products separated by electrophoresis on 0.9% agarose gels. The amount of DNA in the *Eco*RI digest in (B) was approximately one-third the amounts loaded on the gel for the other digests. The gels were blotted onto nitrocellulose and the filters were hybridized with labeled insert from pCHI1. The migration of λ *Hin*dIII DNA fragments in the gels is aligned with each autoradiogram as mol. wt markers.

We conclude that the concerted regulation of the levels of these mRNAs leads to the observed increased biosynthetic enzyme activities (Bell *et al.*, 1984; Cramer *et al.*, 1985a) and, hence, isoflavonoid phytoalexin accumulation in response to pathogens and wounding.

A nearly full-length CHI cDNA clone has been isolated by antibody screening of an expression library and its identity confirmed by hybrid-select translation and *in vitro* transcription/translation. The latter method demonstrated that polypeptides obtained by translation of transcripts of the cDNA insert were precipitated by the CHI antiserum. Several antigenic polypeptides were produced ranging from a dominant 11.4-kd polypeptide to a 19-kd species. One possible explanation for the multiple polypeptides is that the cDNA lacks the initiator methionine and 5′ end of the coding region, but that several internal methionines serve as translation initiation sites with varying efficiencies. It appears that the strongest translation initiation site is located in the last half of the cDNA clone to produce the 11.4-kd polypeptide. Initiation of translation at internal methionines has been reported for several mRNAs (Kozak, 1984; Dixon *et al.*, 1986).

The effect of elicitor on PAL and CHS gene expression in cell cultures has been examined by two methods. Run-off transcription experiments using isolated nuclei have demonstrated increased rates of transcription of these genes within 5 min of elicitor treatment (Lawton and Lamb, 1987). Studies of newly synthesized RNA by pulse-labeling with 4-thiouridine have also demonstrated that elicitor treatment increases the synthesis of PAL and CHS mRNAs (Cramer *et al.*, 1985b; Edwards *et al.*, 1985). Since CHI mRNA was rapidly induced in elicitor-treated cell cultures coordinately with mRNAs encoding PAL and CHS, it is likely that CHI mRNA accumulation results from enhanced transcription of the CHI gene. Post-transcriptional regulation by transient destabilization of these mRNAs is suggested by the dramatic reduction of CHI, PAL and CHS mRNAs to approximately half-maximal levels between 4 and 6 h after elicitor treatment.

In addition to induction by elicitor, CHI mRNA accumulated during the natural interaction between bean plants and *C. lindemuthianum*. The early and late accumulation of CHI mRNA in the incompatible and compatible interactions respectively, closely paralleled the kinetics of PAL and CHS mRNA accumulation previously described (Bell *et al.*, 1986). These data further support a model in which hypersensitive resistance (incompatible interaction) is attributed to early molecular recognition of the pathogen by the plant (Lamb *et al.*, 1986). In this model, this recognition results in rapid transcription of genes encoding particular phenylpropanoid pathway enzymes which leads to isoflavonoid phytoalexin synthesis. In contrast, early recognition of the pathogen does not occur during the compatible interaction. The phenylpropanoid pathway is activated to produce phytoalexins only after substantial fungal growth and subsequent tissue damage associated with the onset of lesion development.

The present study has revealed no evidence for polymorphism of CHI at the gene, mRNA or polypeptide levels. This is in marked contrast to PAL and CHS which are encoded by gene families of 4 and 6−8 members respectively. In both cases, multiple isopolypeptide subunits are synthesized in response to elicitor (Bolwell *et al.*, 1985; Bell *et al.*, 1986; Ryder *et al.*, submitted Cramer and Lamb, unpublished). Emerging evidence indicates that some PAL and CHS genes are differentially regulated by irradiation and biological stress signals such as wounding and infection. In contrast, the single CHI gene is activated by both irradiation (unpublished results) and biological stress. We propose that the CHI gene possesses multiple *cis*-acting regulatory sequences which are independently regulated in response to different environmental signals. Alternatively, a common second messenger system might transduce all these environmental signals and interact with a single regulatory region associated with the CHI gene.

PAL and CHS are the first enzymes of the general phenylpropanoid pathway and flavonoid/isoflavonoid branch pathway respectively, and it is likely that the multi-gene organization of PAL and CHS genes within the bean genome reflects their regulatory roles. For example, it has been shown that PAL isoenzymes possess different enzymatic properties which may be correlated with their differential expression under stress conditions (Bolwell *et al.*, 1985; Liang and Lamb, unpublished). The single CHI enzyme may complement the regulatory functions of PAL and CHS enzymes by virtue of its strong feedback inhibition by products of both the flavonoid and isoflavonoid biosynthetic pathways. We speculate that there has been little selective pressure for duplication and diversification of the CHI gene to perform additional regulatory roles in the phenylpropanoid pathway. Clearly, it will be of considerable interest to examine the structure of the bean CHI gene in comparison with the structures of the PAL and CHS genes in order to define the molecular mechanisms conferring coordinate regulation of these metabolically related genes.

Materials and methods

Plant cell culture and treatment with elicitor

Cells of bean cultivar Canadian Wonder were grown as previously described (Dixon and Bendall, 1978) except that suspensions were cultured in total darkness. *C. lindemuthianum* was grown in liquid culture as previously described (Bailey and Deverall, 1971). Elicitor was prepared using the procedure of Anderson-Prouty and Albersheim (1975). Elicitor was applied to 7- to 10-day-old cell cultures as previously described (Cramer *et al.*, 1985a).

Growth and treatments of plants

Germination and growth of bean cultivar Kievitsboon Koekoek and inoculation of hypocotyls from 8-day-old seedlings with spores of *C. lindemuthianum* races β and γ were as described (Bell *et al.*, 1984). For wound induction, bean (cultivar Tendergreen) seeds were germinated and grown in darkness as previously described (Shields *et al.*, 1982). After 8 days, 10-mm segments were excised from the hypocotyls of half of the seedlings and incubated at 25°C in darkness in 100-mm plastic Petri dishes (~30 segments/dish) containing 10 ml of 50 μg/ml ampicillin solution. The Petri dishes were rotated at 50 r.p.m. for aeration. Wounded and control (intact seedlings retained in the dark) plant material was harvested at various times and frozen in liquid nitrogen.

Isolation of RNA

Total RNA was isolated by two methods. Frozen tissue samples were powdered in a mortar and then homogenized in a phenol:0.1 M Tris, pH 9.0 emulsion as described by Haffner *et al.* (1978). RNA was further purified as described by Palmiter (1974). Alternatively, RNA was extracted using guanidine thiocyanate according to Maniatis *et al.* (1982) as modified by Colbert *et al.* (1983). Polysomal RNA was isolated by a modification (Schroder *et al.*, 1976) of the method of Palmiter (1974). Poly(A)$^+$ RNA was isolated by two cycles of oligo(dT)-cellulose affinity chromatography (Maniatis *et al.*, 1982).

λgt11 cDNA library construction and screening

Poly(A)$^+$ RNA (3 μg) from cell cultures treated with elicitor for 3.5 h was used to direct cDNA synthesis as described (Huynh *et al.*, 1985) except that the reaction included 60 μg/ml actinomycin D and 60 units/ml RNasin. After chromatography over Sephadex G-50, phenol extraction, and ethanol precipitation, second strand synthesis was performed as described by Gubler and Hoffman (1983). The double-stranded cDNA was treated with T4 polymerase to produce blunt ends (Maniatis *et al.*, 1982) and subsequently treated with *Eco*RI methylase according to the protocol of the supplier (Bethesda Research Laboratories). Phosphorylated *Eco*RI linkers were ligated to the cDNA. After *Eco*RI digestion, the products were size-fractionated on a 1% low-melting agarose gel in order to eliminate the cleaved linkers. Gel containing double-stranded cDNA >500 bp was excised, melted and the DNA recovered after phenol extraction and ethanol precipitation. The DNA was ligated to dephosphorylated *Eco*RI-digested λgt11 DNA (Promega Biotec) at a 2:1 molar ratio then packaged *in vitro* according to the protocol of the supplier of the λ packaging mix (Stratagene, Inc.). The library was titered on *Escherichia coli* Y1088 (Young and Davis, 1983a,b) on plates with the top agar containing 160 μg/ml isoproyl-β-D-thiogalactopyranoside (IPTG) and 400 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The library was amplified as plate lysates on Y1088 without IPTG and X-gal. The rabbit antiserum raised against purified bean CHI has been previously characterized (Robbins and Dixon, 1984). Approximately 10^5 recombinant phage in the library prior to amplification were screened with the CHI antiserum using the protocol previously described (de Wet *et al.*, 1984). In this procedure, specifically bound antibodies on the phage lift filters were detected with peroxidase conjugated goat anti-rabbit IgG (BioRad) and the chromogenic peroxidase substrate 4-chloro-1-naphthol.

Hybrid-selection and in vitro translation

Hybrid-selection of 75 μg/ml poly(A)$^+$ RNA from cell cultures treated with elicitor for 3.5 h by filter-bound plasmid DNA was performed as described (Wood *et al.*, 1982) with the following modifications: (i) plasmid DNA (50 μg) was linearized with *Bam*HI; (ii) selected RNA was ethanol precipitated then incubated with DNase 1 (2 units) at 37°C for 1 h in the presence of 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.1 mM DTT, and 60 units/ml RNasin. Following digestion, the samples were phenol–chloroform (1:1) extracted and ethanol precipitated. The RNAs were resuspended in 10 μl of water and 5 μl of each sample was translated *in vitro* in the presence of [^{35}S]methionine in a rabbit reticulocyte translation system (Pelham and Jackson, 1976). After immunoprecipitation with pre-immune serum as described by Lawton *et al.* (1983), the remaining translation products were incubated with anti-CHI serum (5 μl of 1/15 dilution of serum). Immunocomplexes were precipitated with protein A-Sepharose and analyzed by SDS–PAGE as described by Lawton *et al.* (1983) and Robbins and Dixon (1984).

SP6 in vitro transcription

The largest CHI cDNA insert (865 bp) was subcloned into the *Eco*RI site of pSP65 in the appropriate orientation for producing CHI protein coding transcripts. The transcription reaction contained 5 μg of the *Bam*HI linearized plasmid (pCHI1), 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 40 units RNasin, 100 μg/ml BSA, 500 μM ATP, UTP and CTP, 50 μM GTP, 500 μM GpppG, and 30 units SP6 polymerase in 50 μl. After 1 h at 40°C, 5 units DNase were added to the reaction which were then incubated at 37°C for 30 min. The RNA was purified by two phenol–chloroform extractions, Sephadex G-50 chromatography and ethanol precipitation. One tenth of the RNA (~1 μg by spectrophotometric determination) was translated *in vitro*. The products were immunoprecipitated and resolved by SDS–PAGE.

RNA and DNA filter hybridization

Total RNA was size fractionated on formaldehyde agarose gels (Lebrach *et al.*, 1977) and blotted onto nitrocellulose with 10 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were then baked (80°C, 2 h) and prehybridized with a solution containing 50% formamide, 3 × SSC, 60 mM phosphate buffer (pH 6.8), 10 mM EDTA (pH 7.2), 0.2% SDS and 5 × Denhardt solution (Denhardt, 1966). The hybridization solution was the same except for the inclusion of 100 μg/ml of denatured salmon sperm DNA and the cDNA clone inserts labeled by ^{32}P-nick translation. Hybridization occurred at 42°C for 16–24 h. The filters were then washed at 45°C in 0.1 × SSC, 0.1% SDS, air dried and exposed to X-ray film.

Genomic DNA preparations from bean cell cultures and leaves (cultivar Canadian Wonder) were digested with the indicated restriction enzymes, separated by electrophoresis on 0.8% agarose gels and blotted onto nitrocellulose (Southern, 1975). Hybridization conditions were identical to those described above for RNA blots.

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