Phytoalexin synthesis in soybean (*Glycine max*)

Similar time courses of mRNA induction in hypocotyls infected with a fungal pathogen and in cell cultures treated with fungal elicitor

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Rapid, transient increases in mRNA activities related to phytoalexin synthesis were observed after inoculation of soybean seedlings (*Glycine max*) with the fungus, *Phytophthora megasperma* f. sp. *glycinea*, or treatment of cultured soybean cells with an elicitor preparation from the same fungus. The time courses of changes in activity were similar for mRNAs coding for enzymes of general phenylpropanoid metabolism and for chalcone synthase mRNA in both systems. Cloned cDNA was used to demonstrate that the induced changes in chalcone synthase mRNA activity coincided with changes in amount of mRNA. The results suggest that phytoalexin synthesis, one mechanism of induced resistance of plants to pathogens, is regulated by temporary gene activation.

General phenylpropanoid metabolism *Chalcone synthase* *RNA blot hybridization* *Transcriptional control* *Disease resistance*

1. INTRODUCTION

Phytoalexins are antimicrobial substances whose synthesis in higher plants may be caused by infection with potential pathogens or treatment of various plant tissues, including suspension-cultured cells, with pathogen-derived elicitors [1]. The phytoalexins of soybean comprise a mixture of structurally related isoflavonoid derivatives. Fig.1 outlines the biosynthesis of the major component, glyceollin I, from the carbon skeleton of phenylalanine. Important steps are the formation of 4-coumaroyl-CoA, a central intermediate of phenylpropanoid metabolism in higher plants, and of a chalcone, the key intermediate in flavonoid biosynthesis [2].

The rapid induction of two of the enzymes indicated in fig.1, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), has been demonstrated in response to infection of soybean hypocotyls with the fungal pathogen, *P. megasperma* f. sp. *glycinea*, or to treatment of cultured soybean cells with a crude cell-wall preparation (elicitor) from this fungus. In both cases, periods of increases in enzyme activity coincided with high rates of enzyme synthesis, as measured by immunoprecipitation of the in vivo labelled proteins [3,4].

Here, we show that the induced changes in the rates of enzyme synthesis are associated with corresponding changes in extractable mRNA activity. In addition to PAL and CHS mRNAs, the mRNA for the enzyme generating 4-coumaroyl-CoA, 4-coumarate:CoA ligase (4CL), was included in these studies. Furthermore, cloned cDNA complementary to CHS mRNA [5] was used to demonstrate that the timing is the same for changes in amount and activity of CHS mRNA, suggesting that glyceollin synthesis is dependent on pathogen- or elicitor-induced gene expression.

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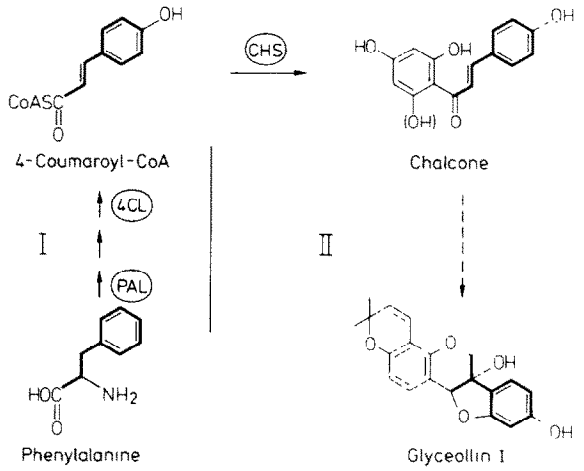

Fig.1. Scheme indicating the positions of 3 central steps in the biosynthesis of glyceollin I, the major isoflavonoid phytoalexin in soybean. The reactions studied here are catalyzed by two enzymes of general phenylpropanoid metabolism (group I), phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL), and one enzyme of the flavonoid pathway (group II), chalcone synthase (CHS). The mode of incorporation of the phenylpropanoid unit from phenylalanine into glyceollin I is indicated by thick lines.

2. MATERIALS AND METHODS

2.1. Materials

L-[^{35}S]Methionine and [^{32}P]dCTP were from Amersham-Buchler, Braunschweig. Protein A–Sepharose CL-4B was obtained from Pharmacia, Freiburg. For nick translation we used the kit from Bethesda Research Laboratories, Gaithersburg, USA. In vitro translation was carried out with the nuclease-treated rabbit reticulocyte lysate purchased from Amersham-Buchler. The elicitor (glucan-rich, autoclaved cell-wall material) was prepared from *P. megasperma* f. sp. *glycinea*, race 1, after growth on liquid medium [6].

2.2. Inoculation of soybean seedlings

Wounds of hypocotyls from 5-day-old soybean seedlings (*G. max* var. Amsoy 71) were inoculated under sterile conditions [3] with mycelium of *P. megasperma*, race 1, which had been grown for 7 days [7]. In control experiments, 2–3 drops of 10 mM sodium phosphate (pH 7.2) were applied to the wounds. For each time point 20 seedlings were incubated in the dark at 25°C in 100% humidity. Hypocotyl segments (2 cm each) were then cut out and frozen in liquid nitrogen.

2.3. Cell cultures

Cell suspension cultures of soybean (*G. max*) were propagated in the dark, treated with elicitor, and harvested as in [4]. Cultures were used when the conductivity of the medium had reached a value of about 2 mS.

2.4. Isolation of RNA

Total RNA was extracted from hypocotyl segments or cells according to [8]. The ethanol-precipitated RNA was washed with 3 M sodium acetate [9], reprecipitated, dissolved in sterile H_2O, and stored at −70°C.

2.5. Translation in vitro

Protein synthesis in vitro was carried out with the nuclease-treated rabbit reticulocyte lysate in the presence of L-[^{35}S]methionine. The translation products were immunoprecipitated by the protein A-immunoadsorption technique [10] with antisera specific for phenylalanine ammonia-lyase (PAL), 4-coumarate:CoA ligase (4CL) or chalcone synthase (CHS) from parsley [11,12]. The antigen–immunoglobulin complexes were separated on a 10% SDS–polyacrylamide slab gel. The radioactively labelled proteins were visualized by fluorography [13]. Relative values for the incorporation rates of radioactivity were obtained by scanning the fluorograms with an LKB laser densitometer scanner.

2.6. RNA blot hybridization

Total RNA (20 μg) was dissolved and denatured in 50% formamide and 6% formaldehyde and separated on a 1.5% agarose gel containing 6% formaldehyde. The RNA was blotted onto Gene Screen (New England Nuclear, Dreieich) and hybridized with cDNA complementary to chalcone synthase mRNA [5], which had been ^{32}P-labelled by nick translation with DNA polymerase I [14,15]. Prehybridization and hybridization were carried out in the presence of 50% formamide and 10% dextran sulfate [16].

3. RESULTS AND DISCUSSION

3.1. mRNA induction in hypocotyls

Hypocotyl wounds of soybean (Amsoy 71) seedlings were inoculated with mycelium of race 1 of *P. megasperma* and the seedlings incubated in the dark for various periods of time. The mycelium was then removed and total RNA isolated from hypocotyl segments at each time point. Portions of the RNA samples were translated in vitro, radioactively labelled PAL, 4CL and CHS immunoprecipitated with specific antisera, the precipitates analyzed by gel electrophoresis, and the relative intensities of bands corresponding to the enzyme subunits determined.

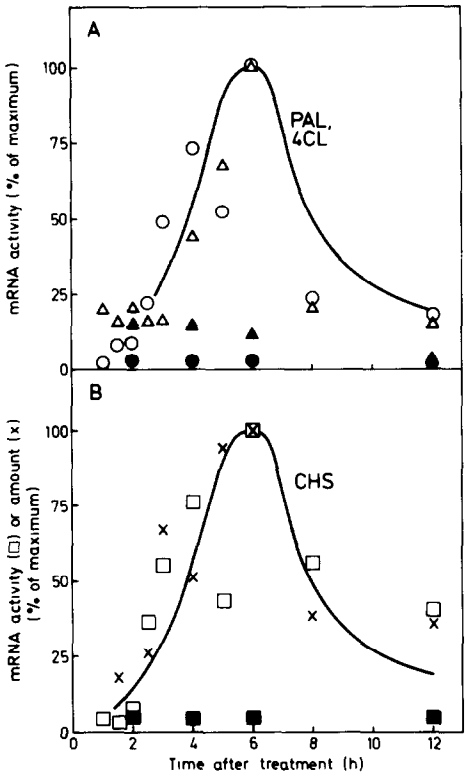

Fig.2. Time courses of mRNA induction in wounded soybean hypocotyls after inoculation with fungal mycelium. Samples were taken from hypocotyl segments at the indicated times after infection (open symbols) or mock inoculation with sterile buffer (closed symbols). (A) Translational activities in vitro of PAL (○,●) and 4CL (△,▲) mRNAs. (B) Translational activity (□,■) and hybridizable amount (×) of CHS mRNA. Identical curves are shown in both panels and are consistent with the respective results within experimental error.

The time courses of changes in PAL, 4Cl and CHS mRNA activities are shown in fig.2A,B. All 3 mRNAs showed similar patterns of changes and reached peaks in activity around 6 h after inoculation of the seedlings. This result is in agreement with previous findings by authors in [3] who demonstrated, by pulse-labelling of infected plants with L-[^{35}S]methionine and subsequent immunoprecipitation, that the rate of PAL synthesis in vivo was also maximal around 6 h after inoculation of soybean seedlings with race 1 of *P. megasperma*.

The observed changes in mRNA translational activity can be due to translation control of an already present mRNA or to changes in its amount. To distinguish between these possibilities, we carried out RNA blot hybridization experiments with electrophoretically separated RNA from the same samples as used above. ^{32}P-labelled cDNA probes for PAL, 4CL and CHS mRNAs from cultured parsley cells [5,17] were used for separate hybridizations. Measurable amounts of radioactivity were obtained only for CHS mRNA. This mRNA migrated to the expected position corresponding to a length of about 1700 nucleotides [5]. Fig.2B shows that the relative changes in the hybridizable amount of CHS mRNA after infection of soybean seedlings with the fungus coincided with the

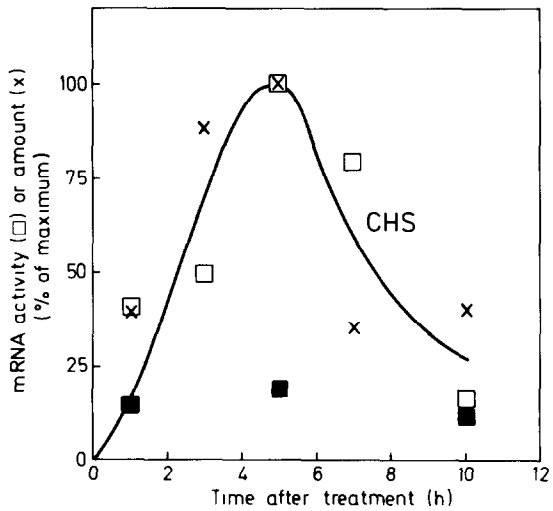

Fig.3. Time course of CHS mRNA induction in cultured soybean cells after treatment with fungal elicitor. Symbols for translational activity (□,■) and hybridizable amount (×) as in fig.2, except that control experiments (■) were carried out with untreated samples.

changes in CHS mRNA activity. It is unknown whether under our experimental conditions the amounts of PAL and 4CL mRNAs were too small to be detected or cross-hybridization of the mRNAs from soybean with cDNAs from parsley was too weak.

3.2. mRNA induction in cultured cells

It has recently been shown [18] that treatment of soybean cell suspension cultures with an elicitor preparation from cell walls of *P. megasperma* induced large, transient increases in PAL and CHS mRNA activities. Highest activities occurred between 5 and 7 h after addition of the elicitor. The time course of elicitor-induced changes in CHS mRNA activity, shown here in fig.3, confirms this previous observation.

Hybridization experiments analogous to those described above for mRNA from soybean seedlings were also carried out with the mRNA from cultured cells. Again, significant amounts were only found for CHS mRNA. Within experimental error, the elicitor-induced changes coincided for amount and activity of CHS mRNA and were maximal around 5 h after the onset of induction.

4. CONCLUSIONS

For biochemical studies of plant–pathogen interactions it is of great value to use systems of reduced complexity, particularly cell suspension cultures in combination with elicitors. With this model system it is possible to measure the responses of a largely homogeneous cell population under controlled conditions. Considering the inevitable limitations of such reduced systems [19], it was interesting to note that the timing of changes in mRNA activities coding for enzymes of general phenylpropanoid metabolism (PAL, 4CL) and for the key enzyme of the flavonoid pathway (CHS) was very similar for infected hypocotyls (fig.2) and elicitor-treated cell cultures (fig.3 and [18]).

Although similar time courses of changes in these mRNA activities have now been demonstrated in both experimental systems, coincidence of the changes in amount and activity could be determined only for CHS mRNA. This mRNA was present in sufficient amounts to enable RNA blot hybridization experiments. However, the same elicitor as used here has been shown to induce both the amounts and activities of PAL and 4CL mRNAs in cultured parsley cells [17]. Moreover, the induction in parsley cells was associated with large, transient increases in the rates of de novo synthesis of these mRNAs, i.e., with gene activation (submitted). On the other hand, the phytoalexins in parsley are furanocoumarins, whose synthesis involves PAL and 4CL but not CHS. Since we have now shown that CHS was induced in the two soybean systems through corresponding changes in amount and activity of CHS mRNA, it is very likely that in soybean, as in parsley, phytoalexin synthesis as one of the major defense reactions against pathogens is controlled at the level of gene transcription.

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