MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 2, No. 6, pp. 363-368, 1989

# Characterization of Elicitor-Induced Defense Responses in Suspension-Cultured Cells of *Arabidopsis*

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Received 10 May 1989. Accepted 26 July 1989.

**When suspension-cultured cells of *Arabidopsis* (ecotype Fi-3) were treated with crude preparations of the bacterial pectin-degrading enzyme α-1,4-endopolygalacturonic acid lyase (PGA lyase), they expressed a number of putative defense responses including increased levels of several enzymes involved in phenylpropanoid biosynthesis. Phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL) were transiently induced with similar induction kinetics and reached maximum levels at 8 to 10 hr after elicitor treatment. Caffeic acid *O*-methyl transferase (CMT) and peroxidase had induction kinetics distinct from those observed for PAL and 4CL; maximum levels of these two enzymes were observed approximately 24 hr after elicitor treatment and were maintained for at least 48 hr. The transient increases in PAL and 4CL enzyme activities were preceded by transient increases in the steady-state levels of their mRNAs, which peaked approximately 3 hr after elicitor treatment. Cell cultures treated with PGA lyase also had increased steady-state levels of mRNAs of β-1,3-glucanase, another putative plant defense response. These results demonstrate that the overall response of *Arabidopsis* cells to elicitor treatment is very similar to that observed in other cell culture systems and provides the first indication as to the nature of some of the defense responses elaborated by *Arabidopsis*. This is an important first step in developing *Arabidopsis* as a model system for studying plant-pathogen interactions.**

*Additional keywords*: phenylpropanoid metabolism, Pmg elicitor.

Plants defend themselves from attempted infection by elaborating a number of diverse defense responses (Bell 1981; Hahlbrock and Scheel 1987). This array of defenses includes several inducible responses that are activated by microorganisms or by specific molecules (elicitors) derived from microorganisms and plant cell walls (Darvill and Albersheim 1984). These inducible defense responses include the synthesis and accumulation of polyphenolic lignins, hydroxyproline-rich glycoproteins, hydrolytic enzymes such as β-1,3-glucanase and chitinase, and antimicrobial compounds called phytoalexins (Kombrink *et al.* 1986; Collinge and Slusarenko 1987).

Studies by a number of researchers using biochemical and molecular biological approaches have shown that the activation of these presumptive defense responses is often correlated with disease resistance in specific plant-microorganism interactions (Bell *et al.* 1986; Hahn *et al.* 1985; Jahnen and Hahlbrock 1988; Habereder *et al.* 1989). However, because these data are strictly correlative, it has been difficult to demonstrate clearly that a particular response is required for the plant to be resistant to infection. To overcome some of the limitations of these correlative approaches, we have initiated studies aimed at using a combination of genetic and molecular biological approaches to study disease resistance in *Arabidopsis thaliana* (L.) Heynh. The development of this system will allow both biochemical and molecular biological experiments to be combined with genetic analyses of specific defense response mutations, thus making it possible to more clearly define which putative defense responses are truly important in preventing microbial infection.

*A. thaliana*, a small crucifer that has received considerable attention as a model plant system, offers a number of advantages for genetic and molecular biological studies of disease resistance in plants (Meyerowitz 1987). However, a disadvantage of *A. thaliana* is that there are no characterized pathogens and no reports concerning the defense responses which this plant has when confronted with a potential pathogen. Therefore, as a first step in developing *A. thaliana* as a model system for studying plant-pathogen interactions, we established a suspension culture system in which putative defense responses could be induced by elicitor treatments. This approach has been used successfully to identify putative defense genes in several plant species (Ebel *et al.* 1984; Edwards *et al.* 1985; Kombrink *et al.* 1986), and recent studies have shown that the response of plant cells to elicitor treatment is very similar to that observed during microbial infection (Fritzemeier *et al.* 1987; Habereder *et al.* 1989).

In this report, we demonstrate that several responses previously associated with disease resistance in other plants are induced in suspension-cultured *Arabidopsis* cells treated with the bacterial elicitor α-1,4-endopolygalacturonic acid lyase (PGA lyase). These responses include the induction of enzymes involved in phenylpropanoid metabolism and increased levels of mRNAs encoding β-1,3-glucanase.

## MATERIALS AND METHODS

**Cell cultures.** Seeds of different ecotypes of *A. thaliana* were obtained from the Arabidopsis Information Service

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(Frankfurt, West Germany). Callus cultures were derived from leaf pieces of aseptically grown seedlings of ecotypes Col-0 (Columbia), Co-0, La-0, No-0, and Fi-3 placed on Gamborg's B5 medium (Gibco Laboratories, Grand Island, NY) containing 1 mg/L of 2,4-dichlorophenoxyacetic acid (1-B5) solidified with 0.8% agar. Callus cultures were maintained on 1-B5 plates in the dark at 24° C and subcultured every 3 to 4 wk. Suspension cultures were initiated from callus of ecotype Fi-3 because this callus was very soft and friable in contrast to the very hard callus obtained from the other ecotypes. Pieces of callus were placed in liquid 1-B5 medium and maintained in the dark at 24° C on a rotary shaker operating at 110 revolutions per minute. Suspension cultures were subcultured every 7 days by transferring 5 ml of culture to 40 ml of fresh medium. Larger cultures were started by transferring 40 ml of a 7-day-old culture into 400 ml of fresh medium. The first experiments were conducted 2 mo after the suspension cultures were initiated.

After the experiments reported in this paper were completed, we obtained evidence that ecotype Fi-3 may actually be a polyploid strain of *A. thaliana* or a hybrid between *A. thaliana* and another closely related *Arabidopsis* species (D. Voytas, K. R. Davis, E. Schott, F. M. Ausubel, and I. Al-Shebaz, unpublished data). This conclusion was based on the following observations. DNA blots of Fi-3 genomic DNA probed with *A. thaliana* genomic sequences of alcohol dehydrogenase (Chang and Meyerowitz 1986), the retroviral-like element Tal (Voytas and Ausubel 1988), and chalcone synthase (Feinbaum and Ausubel 1988) had a restriction pattern identical to that observed with the other 16 *A. thaliana* ecotypes tested; Fi-3 DNA probed with an *A. thaliana* phenylalanine ammonia-lyase (PAL) probe (K. R. Davis, unpublished) gave several bands in addition to those observed with the other ecotypes. Cytogenetic analysis of root tip squashes clearly showed that Fi-3 cells have more than 10 chromosomes; however, an accurate determination of the total number was not possible. Morphological characteristics indicated that Fi-3 may be an interspecific hybrid.

**Elicitor preparation and treatment of cell cultures.** Crude PGA lyase preparations were obtained by dialyzing culture filtrates of *Erwinia carotovora* pv. *carotovora* (ATCC No. 495) grown on pectin-containing medium (Davis *et al.* 1984) against 5 mM Tris-HCl (pH 8.5) containing 1 mM $CaCl_2$. Previous studies have demonstrated that the major, if not only, elicitors in this preparation are two isozymes of PGA lyase (Davis *et al.* 1984). PGA lyase activity was measured spectrophotometrically (Davis *et al.* 1984) and preparations were filter-sterilized before being added to cultures.

A crude fungal elicitor obtained from cell walls of *Phytophthora megasperma* f. sp. *glycinea* was prepared as described previously (Kombrink and Hahlbrock 1986) and kindly provided by Erich Kombrink.

Experimental treatments were conducted by continuously exposing 6- or 7-day-old 400-ml cultures to elicitors as previously described for parsley cell cultures (Davis and Hahlbrock 1987). Cells were harvested by filtration, frozen with liquid nitrogen, and stored at −70° C. All experiments were repeated at least twice; the data presented are from representative experiments unless otherwise indicated.

**Determination of enzyme activities.** Cells were extracted for enzyme activities, and protein concentrations were determined as previously described (Kombrink and Hahlbrock 1986). PAL and 4-coumarate:CoA ligase (4CL) activities were determined using standard spectrophotometric assays (Kombrink and Hahlbrock 1986). Caffeic acid *O*-methyl transferase (CMT) activity was measured using a modification of a previously described assay (Hauffe *et al.* 1986). Reaction mixtures contained 5 μl of protein extract (approximately 10 μg of protein), 1.5 mM $MgCl_2$, 100 mM $KPO_4$ (pH 7.5), 1 mM caffeic acid, 10 μM [$^{14}CH_3$]*S*-adenosyl-L-methionine (New England Nuclear, Boston, MA, 58.6 mCi/mmole), and 35 mM ascorbic acid in a total volume of 70 μl. Reaction mixtures were incubated at 30° C for 30 min, and reactions were terminated by adding 20 μl of 4 N HCl. Reaction mixtures were extracted with 150 μl of ethyl acetate, and the amount of radioactivity present in 50-μl aliquots of the extracts was determined by liquid scintillation spectrometry. Peroxidase activity was determined by measuring the change in the $A_{470}$ of a reaction mixture containing 1 μl of protein extract (approximately 2 μg of protein), 100 mM sodium acetate (pH 6.0), 10 mM guaiacol, and 10 mM $H_2O_2$ in a total volume of 3.0 ml. Assays were incubated at 30° C, and the $A_{470}$ was measured during the first 2 min of the reaction. Assays were done in duplicate and averaged; duplicate assays varied by 10% or less.

**RNA isolation and analysis.** Total RNA was isolated by a modified phenol-sodium dodecyl sulfate (SDS), LiCl precipitation, procedure (Ausubel *et al.* 1987). RNA blots were prepared by electrophoresis of 10 or 15 μg of RNA in formaldehyde-agarose gels as previously described (see an article on the comparison of glyoxal and formaldehyde gels for sizing mRNAs published in Vol. 8, pages 5 and 6, of Focus, a publication of Bethesda Research Laboratories, Gaithersburg, MD), and capillary transfer of separated RNAs onto nylon membranes (Gene Screen, New England Nuclear) was conducted using 10× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate). RNA was covalently attached to the membranes by UV-crosslinking and baking at 80° C for 2 to 3 hr. Gels were stained with ethidium bromide before capillary transfer to visualize the rRNA bands.

Filters were prehybridized in 1 M NaCl, 1% SDS, 10% dextran sulfate, and 50% formamide for 2–5 hr at 37° C. Heat-denatured, nick-translated plasmids or gel-purified inserts labeled by a random priming reaction (Boehringer Mannheim, Indianapolis, IN) were used as probes and were added to the prehybridization mixture to a final concentration of $10^5$ cpm/ml in the presence of 100 μg/ml of salmon testes DNA (Sigma, St. Louis, MO). Hybridizations were conducted at 37° C for 16–20 hr. Filters were then washed at 50° C for approximately 1 hr with two changes of 2× SSC containing 1% SDS. Washed filters were blotted dry, wrapped in plastic wrap, and used for autoradiography. In cases in which the filter was reprobed, the filter was first stripped by placing in boiling 5 mM Tris-HCl (pH 7.5) and incubating with constant agitation at 65° C for 1 hr.

The following cDNA probes were used. A bean PAL cDNA (pPAL5) was obtained from C. Cramer and contains a 1.5-kilobase (kb) insert (Edwards *et al.* 1985); the potato 4CL cDNA was provided by K. Hahlbrock and contains a nearly full-length insert of 2.0 kb (Fritzemeier *et al.* 1987); the tobacco β-1,3-glucanase cDNA (pGL43) was provided by F. Meins and contains a 1-kb insert (Mohnen *et al.* 1985); and the tobacco PR1a cDNA (pNt$^{SNN}$cPR1a/35) was provided by U. Pfitzner and contains a full-length insert of 0.8 kb (Pfitzner and Goodman 1987).

## RESULTS

**Induction of enzymes involved in phenylpropanoid biosynthesis.** Previous studies have demonstrated that phenylpropanoid biosynthesis is often induced in plant cell cultures treated with elicitors (Ebel *et al.* 1984; Cramer *et al.* 1985; Kombrink *et al.* 1986). To determine if this is also true for *Arabidopsis*, Fi-3 cell cultures were treated with two different microbial elicitors, the bacterial pectin-degrading enzyme PGA lyase and cell wall components from the phytopathogenic fungus *P. m.* f. sp. *glycinea* (Pmg elicitor). Cell cultures treated with either crude PGA lyase preparations or Pmg elicitor had increased levels of PAL activity (Figs. 1A and 1B). The level of PAL activity observed in cells 10 to 12 hr after elicitor treatment increased in a dose-dependent manner between 10 and 100 munits/ml of PGA lyase and 2 to 10 μg/ml of Pmg elicitor.

4CL enzyme activity was induced coordinately with PAL activity in cultures treated with PGA lyase (Fig. 1C). The induction of PAL and 4CL activities in cell cultures 10 to 12 hr after treatment with 50 munits/ml of PGA lyase varied in different experiments from 8- to 30-fold and 3- to 6-fold, respectively, above control levels; this variation was due primarily to the variability of these enzyme activities in the control cell cultures. CMT and peroxidase activities were also induced by PGA lyase in a dose-dependent manner similar to that observed for PAL and 4CL. Concomitant with the induction of enzymes involved in phenylpropanoid biosynthesis, elicitor-treated cells became brown and showed a decreased growth rate, as determined by measuring fresh weights of cells 48 hr after treatment (data not shown). Preliminary experiments indicated that the induction of PAL and 4CL activities in cell cultures of ecotype Columbia treated with PGA lyase was similar to that observed in the Fi-3 cultures.

The analysis of the induction kinetics of PAL, 4CL, CMT, and peroxidase activities in cell cultures treated with 50 munits/ml of PGA lyase is shown in Figure 2. PAL and 4CL activities were transiently induced in parallel by PGA lyase, reaching maximum activities 6 to 8 hr after elicitor treatment (Figs. 2A and 2B). PAL activity then decreased to control levels by 24 to 32 hr after elicitor treatment, whereas 4CL activity decreased more slowly, with a twofold induction over control levels still evident 48 hr after elicitor treatment. At 8 hr after elicitor treatment, PAL and 4CL activities were induced sevenfold and fivefold, respectively, above that observed in cell cultures treated with heat-inactivated PGA lyase preparations.

CMT and peroxidase activities had similar induction kinetics that were clearly distinct from those observed for

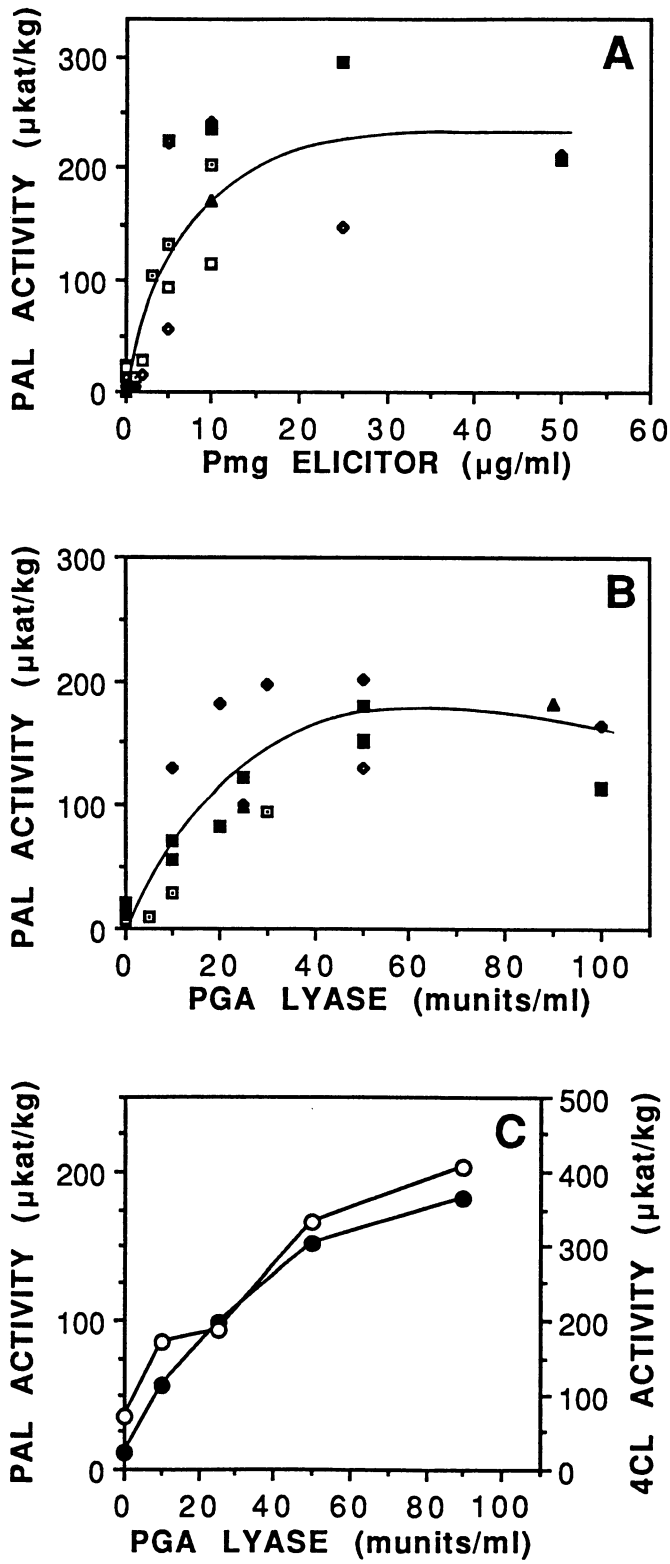


**Fig. 1.** Induction of phenylalanine ammonia-lyase (PAL) activity in *Arabidopsis* cell cultures treated with different concentrations of either crude cell wall components from *Phytophthora megasperma* f. sp. *glycinea* (Pmg elicitor) (**A**) or α-1,4-endopolygalacturonic acid lyase (PGA lyase) (**B**). The symbols represent determinations from separate experiments done over the course of several months. The coordinate induction of PAL (●) and 4-coumarate:CoA ligase (4CL) (○) by different concentrations of PGA lyase is shown in panel **C**. Cells were harvested 10 to 12 hr after elicitor treatment and analyzed as described in the text.

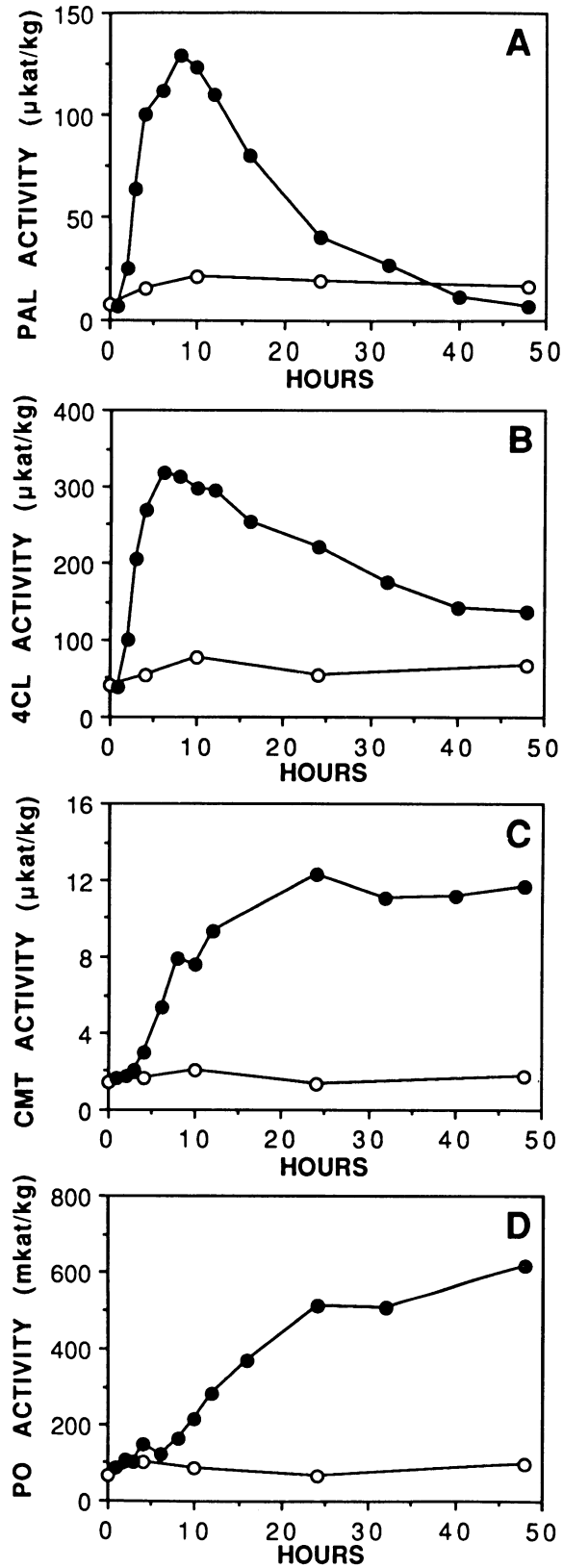


**Fig. 2.** Time courses for the induction of phenylalanine ammonia-lyase (PAL) (**A**), 4-coumarate:CoA ligase (4CL) (**B**), caffeic acid *O*-methyl transferase (CMT) (**C**), and peroxidase (PO, **D**) activities in *Arabidopsis* cell cultures treated with 50 munits/ml of α-1,4-endopolygalacturonic acid lyase (PGA lyase) (●) or heat-inactivated PGA lyase (○). Enzyme activities were determined as described in the text.

PAL and 4CL (Figs. 2C and 2D). CMT and peroxidase activities increased more slowly than PAL and 4CL activities, reaching maximum levels approximately 24 hr after elicitor treatment. The induced levels of these enzymes were maintained for at least 48 hr after elicitor treatment. At 24 hr after elicitor treatment, CMT and peroxidase activities were induced ninefold and eightfold, respectively, above that observed in control cell cultures.

**Elicitor induced changes in levels of PAL, 4CL, and β-1,3-glucanase mRNAs.** To determine if the increases in PAL and 4CL activities were correlated with increased levels of mRNAs encoding these enzymes, total RNA was isolated from cells at various times after treatment with 50 munits/ml of PGA lyase. These RNAs were subjected to RNA blot analysis using cDNAs from bean (PAL) and potato (4CL) as probes. Specific mRNAs that were induced by PGA lyase were detected with the heterologous PAL and 4CL probes (Figs. 3A and 3B). The sizes of the mRNAs detected by the PAL and 4CL probes were 2.8-kb and 2.0-kb, respectively. These sizes are very similar to the sizes of the corresponding PAL and 4CL mRNAs in bean and potato (Edwards *et al.* 1985; Fritzemeier *et al.* 1987). These putative *Arabidopsis* PAL and 4CL mRNAs were rapidly and coordinately induced in elicitor treated Fi-3 cell cultures, reaching maximum levels at 3 hr after elicitor treatment (approximately 30- and 100-fold induction for PAL and 4CL, respectively). The levels of these mRNAs declined after 3 hr, reaching control levels at 15 to 20 hr after elicitor treatment.

To determine whether other putative defense responses were induced in cultures treated with PGA lyase, a similar RNA blot analysis was conducted using tobacco cDNAs of β-1,3-glucanase and PR1a as probes. The tobacco β-1,3-glucanase cDNA hybridized to a specific *Arabidopsis* mRNA of 1.7 kb (Fig. 3C), which is very similar to the size of the corresponding tobacco mRNA (Mohnen *et al.* 1985). This mRNA was induced in PGA lyase treated cultures with induction kinetics distinct from that observed for PAL and 4CL. The putative β-1,3-glucanase mRNA was transiently induced after a lag period of approximately 3 hr, reaching maximum levels at approximately 8 to 16 hr, followed by a return to control levels by 24 hr after elicitor treatment. This putative β-1,3-glucanase mRNA was induced threefold above control levels during the period of maximum induction. The tobacco PR1a cDNA hybridized to a specific mRNA of 0.8 kb, which is similar to the size reported for the tobacco PR1a mRNA (Pfitzner and Goodman 1987). The levels of this mRNA varied significantly in control cells and its induction by PGA lyase was not reproducible; in some cases a twofold induction was observed with induction kinetics similar to that shown by the β-1,3-glucanase mRNA (data not shown).

## DISCUSSION

We have demonstrated that cultured cells of *Arabidopsis* respond to treatment with two microbial elicitors, PGA lyase and crude fungal cell wall components. The response of the *Arabidopsis* suspension cultures to treatment with PGA lyase was examined in more detail and was found to be very similar to the putative defense responses

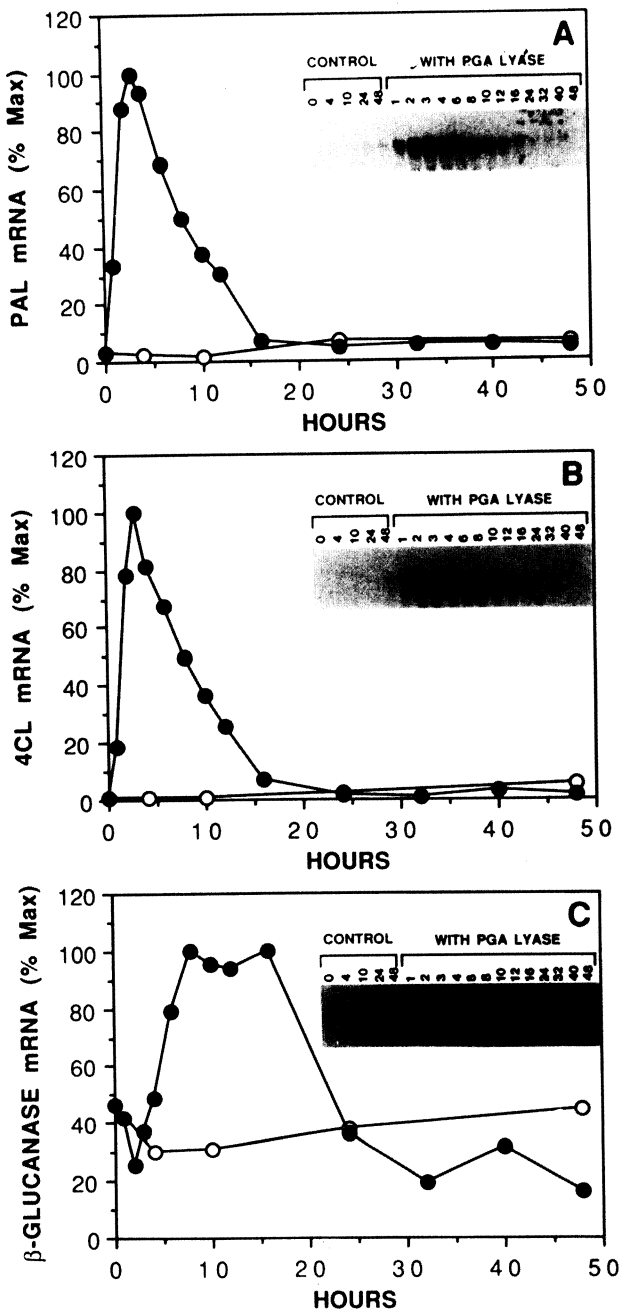


**Fig. 3.** Time courses for the induction of phenylalanine ammonia-lyase (PAL) (**A**), 4-coumarate:CoA ligase (4CL) (**B**), and β-1,3-glucanase (**C**) mRNAs in cultured *Arabidopsis* cells treated with 50 munits/ml of α-1,4-endopolygalacturonic acid lyase (PGA lyase) (●) or heat-inactivated PGA lyase (○). Total RNA was isolated from cells, and 10-μg portions were used to prepare RNA blots as described in the text. Ethidium bromide staining of the agarose gel before transfer revealed that the amount of rRNA present in each lane was very similar. The RNA blot was probed with labeled cDNA inserts; the data shown were obtained from the same RNA blot that had been stripped after each hybridization experiment. RNA that hybridized to the probes was detected by autoradiography and quantitated by scanning the densitometry of several different exposures of the blot. The densitometry data are expressed relative to the maximum level observed with a particular probe. Similar results were obtained in two other experiments in which fewer time points were examined (data not shown).

previously described in parsley cell cultures (Davis and Hahlbrock 1987).

PGA lyase induced a rapid, transient induction of PAL and 4CL mRNA levels, which was followed by a transient increase in these two enzyme activities. The results, which are consistent with the regulation of these genes in other plants (Edwards *et al.* 1985; Fritzemeier *et al.* 1987; Habereder *et al.* 1989), suggest that PAL and 4CL are regulated at the level of mRNA. Concomitant with the increase in PAL and 4CL activities were increases in the activities of CMT and peroxidase in elicitor-treated cultures. These results indicate that, as it is for many other plants, phenylpropanoid biosynthesis may be an important component of the defense response of *Arabidopsis*. The nature of the phenylpropanoid products produced as defense compounds by *Arabidopsis* remains to be described. Preliminary data from experiments using genomic sequences of the *A. thaliana* chalcone synthase gene as a probe (Feinbaum and Ausubel 1988) indicate that chalcone synthase mRNA is not induced in cultured *Arabidopsis* cells by elicitor treatment (R. Feinbaum, K. R. Davis, and F. M. Ausubel, unpublished data). This suggests that flavonoid derivatives are not produced as defense compounds in *Arabidopsis*. The observed induction of CMT and peroxidase activities suggests that ligninlike compounds may be involved in the defense response.

We also examined the induction of two other putative defense responses, β-1,3-glucanase and PR1a synthesis, in elicitor-treated *Arabidopsis* cells. RNA blot analyses demonstrated that increased levels of β-1,3-glucanase mRNA were induced by PGA lyase, whereas induction of PR1a was marginal and not reproducible. The induction kinetics of β-1,3-glucanase mRNA were distinct from those observed for PAL and 4CL mRNAs. This demonstrates that the differential regulation of various defense genes which is observed in other plants also occurs in *Arabidopsis*.

As discussed previously in the text, after the completion of studies with ecotype Fi-3 cell cultures, we determined that Fi-3 may be a polyploid strain of *A. thaliana* or a hybrid between *A. thaliana* and another closely related *Arabidopsis* species. Therefore, additional experiments were conducted to determine whether the induction of PAL and 4CL enzyme activities in *A. thaliana* race Columbia cell cultures treated with PGA lyase was similar to that observed in the Fi-3 cultures. Preliminary studies showed that the induction of PAL and 4CL by PGA lyase was similar in both cell culture systems, indicating that the results obtained using the Fi-3 cells are likely to be representative of the responses of other *A. thaliana* ecotypes.

In summary, the results of this study clearly demonstrate that the response of *Arabidopsis* cell cultures to elicitor treatment is similar to that observed in other plant cell culture systems. This would suggest that the regulation of putative defense responses of *Arabidopsis* is similar, if not identical, to the regulation of defense genes in other plant species. Thus, the results of our current studies concerning the induction of defense responses of *Arabidopsis* inoculated with phytopathogenic bacteria will likely provide insight into the regulation of defense responses in economically important plant species.

## ACKNOWLEDGMENTS

We thank Carol Cramer, Klaus Hahlbrock, Ursula Pfitzner, and Fred Meins for providing cDNA clones. We also thank Rachel Hyde for assistance in preparing the manuscript.

This work was supported by a grant from Hoechst AG to Massachusetts General Hospital.

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