INDUCTION OF PHENYLPROPANOID METABOLISM IN ELICITOR-TREATED HYBRID POPLAR SUSPENSION-CULTURED CELLS

by

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B.Sc. hon., The University of British Columbia, 1990

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE MASTER

OF SCIENCE DEGREE

in

THE FACULTY OF GRADUATE STUDIES

(Botany Department)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 1991

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Date April 30, 1991

DE-6 (2/88)

ABSTRACT

Induction of phenylpropanoid metabolism in many plants is associated with the induction of plant defence responses. Among these are the accumulation of phenylpropanoid-derived phytoalexins, increase in lignification around infected sites, and the accumulation of wall-bound phenolic compounds. I show in this work, that H11 hybrid cell suspension cultures when treated with either of three elicitors respond with an increase in phenylpropanoid metabolism. Activation proceeds rapidly from PAL and 4CL mRNA accumulation, to a massive increase in extractable PAL enzyme activity and finally there is accumulation of specific phenolic compounds in the cell extracts, culture filtrates, and cell walls. In addition, elicitor treatment causes cells to turn brown, indicative of phenolic compound accumulation. As in other plants, induction is dependent on culture age, is dose dependent, and the kinetics of induction is the same with all three elicitors. Based on the previously established mode of action of PGA lyase as an elicitor, it is concluded that in poplar, as in other plants, defence responses can be induced by elicitors from both fungal and plant cell wall origin. These results illustrate the successful use of plant suspension cultures as a simplified system to study inducible defence responses. In addition, and consistent with the ubiquitous nature of phenolics in poplar, phenylpropanoid metabolism may play an important role in plant defence responses in this species.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Carl Douglas for giving me the opportunity of doing a master of science degree under his supervision, for his guidance in developing this thesis research, and for the financial assistance that he provided over the course of two years. I am greatly indebted to Gopal Subramaniam for his assistance in the technical aspects of this research, and Brian Ellis, Mark Bernards and Malcolm Campbell for their assistance with the HPLC work. I also wish to thank my lab mates, Mary Ellard, Beth Molitor, Kadi Hauffe, Frank Williams, and Susanna Reinold for making my first experience working in a lab a lot of fun.

I am also greatly indebted to the Botany Department headed by Dr. Anthony Glass for accepting me into the masters program and providing me with financial assistance.

INTRODUCTION

Plants often respond to pathogen attack by producing a barrage of compounds designed to restrict the growth of the pathogen and thus prevent further colonization of healthy plant tissue. These inducible responses include the production of low molecular weight antimicrobial compounds called phytoalexins (Dixon, 1986; Paxton, 1981), deposition of lignin and other cell wall-bound phenolics around the site of infection (Bolwell et al,1985; Grisebach,1981), deposition of hydroxyproline-rich glycoproteins, and localized synthesis of proteins such as ß-1,3-glucanases and chitinases which degrade fungal cell walls (Hedrick et al., 1988; Showalter et al., 1985). The induction of these responses is often associated with a localized plant cell death around the site of infection, thus effectively isolating the pathogen. This is known as the hypersensitive response (HR). Two groups of genes are generally thought to mediate this type of active resistance (Callow, 1987): plant 'recognition' genes which interact with products of pathogen avirulence genes, and host 'response' genes that are triggered by the act of recognition and lead to the expression of some facet of the overall resistance response, such as phytoalexin and lignin synthesis. Very little is known about the signal transduction steps from recognition leading to the activation of plant defence genes.

Recognition is thought to be mediated by receptors on the plasma membrane (Callow, 1987; Schmidt and Ebel, 1987) which recognize specific molecules of plant or pathogen origin and thus trigger the defence responses. Many compounds have been shown to induce (or elicit) defence responses in cell cultures and intact tissue (Templeton and Lamb, 1988). They include glycoproteins, simple and complex carbohydrates and certain fatty acids (Darvill

and Albersheim, 1984; Dixon, 1986). Fungal culture fluids and the fraction heat-released from mycelial cell walls have been used as elicitors (Darvill and Albersheim, 1984). Bacterial and fungal enzymes which release endogenous plant cell wall fragments have also been reported to induce defence responses (Bruce and West, 1989; Davis et al., 1984; Davis and Hahlbrock, 1987; Davis and Ausubel, 1989). Most attempts to correlate elicitor activity with species or race-cultivar specificity have failed. However, in tomato infected with *Cladosporium fulvum*, a necrosis-inducing peptide has been isolated from intercellular spaces of infected leaves which exhibits appropriate race-cultivar specificity (De Wit et al., 1985). The study of elicitor-treated plant cell cultures has provided valuable information on the mechanisms regulating the elaboration of inducible defence compounds. Only the induction of phenylpropanoid phytoalexins and lignin will be addressed here.

In general, elicitors cause a rapid but transient change in the synthesis of defence-related cellular proteins, which is preceded by increases in mRNA amounts (Bolwell et al., 1985; Bruce and West, 1989; Davis and Hahlbrock, 1987; Davis and Ausubel, 1989; Chapell and Hahlbrock, 1984; Cramer et al., 1985). Some elicitor-inducible genes encode enzymes of general phenylpropanoid metabolism (GPM) and specific phenylpropanoid branch pathways, such as those leading to lignin (Bruce and West, 1989; Grand et al., 1987), isoflavanoids, (Bolwell et al., 1985; Cramer et al., 1985), and furanocoumarin synthesis (Chapell and Hahlbrock, 1984). Phenylpropanoid metabolism is also induced in pathogen-infected tissue (Hahlbrock and Scheel,1989). Thus, phenylpropanoid metabolites appear to play important roles in plant defence against pathogens (Hahlbrock and Scheel, 1989).

As well as their roles as defence compounds, phenylpropanoid metabolites play important and varied roles during normal plant development.

GPM is required to supply precursors for all specific branch pathways (Fig. 1). Phenylpropanoids serve as flower pigments, UV protectants, insect repellants, and signal compounds involved in plant-microbe interactions (Hahlbrock and Scheel, 1989). In addition, they serve as complex polymeric components of surface and support structures as suberin, lignin and other cell-wall components.

The first step of general phenylpropanoid metabolism is the deamination of phenylalanine by the enzyme phenylalanine ammonia lyase (PAL) to form cinnamic acid. Phenylalanine itself is derived from the shikimate pathway. Cinnamate 4-hydroxylase (C4H) catalyses the hydroxylation of cinnamic acid to yield 4-coumarate. 4-coumarate can be hydroxylated and methylated at various positions before being acted upon by 4-coumarate:CoA ligase (4CL). The products are the CoA thioesters of the corresponding cinnamic acids. The individual branch pathways of phenylpropanoid metabolism derive their basic phenylpropanoid units from these core reactions of GPM. The precise branch point is unknown in many cases, but the involvement of the last steps of the core reactions for the formation of Coenzyme A esters of cinnamic acid derivatives by 4CL, has been demonstrated for the lignin and flavonoid pathways (Hahlbrock et al., 1989).

The cell walls are a major site in which products of general phenylpropanoid metabolism accumulate (Vance et al., 1980). These metabolites are found in the form of monomers such as wall-esterified and ether-linked hydroxycinnamic acids, dimeric forms of ferulic and *p*-coumaric acid, and polymers such as lignin and suberin. These substances are normally an integral part of the cell structure (Vance et al., 1980). Lignins are required mainly for mechanical support, but both they and suberin can act as barriers to

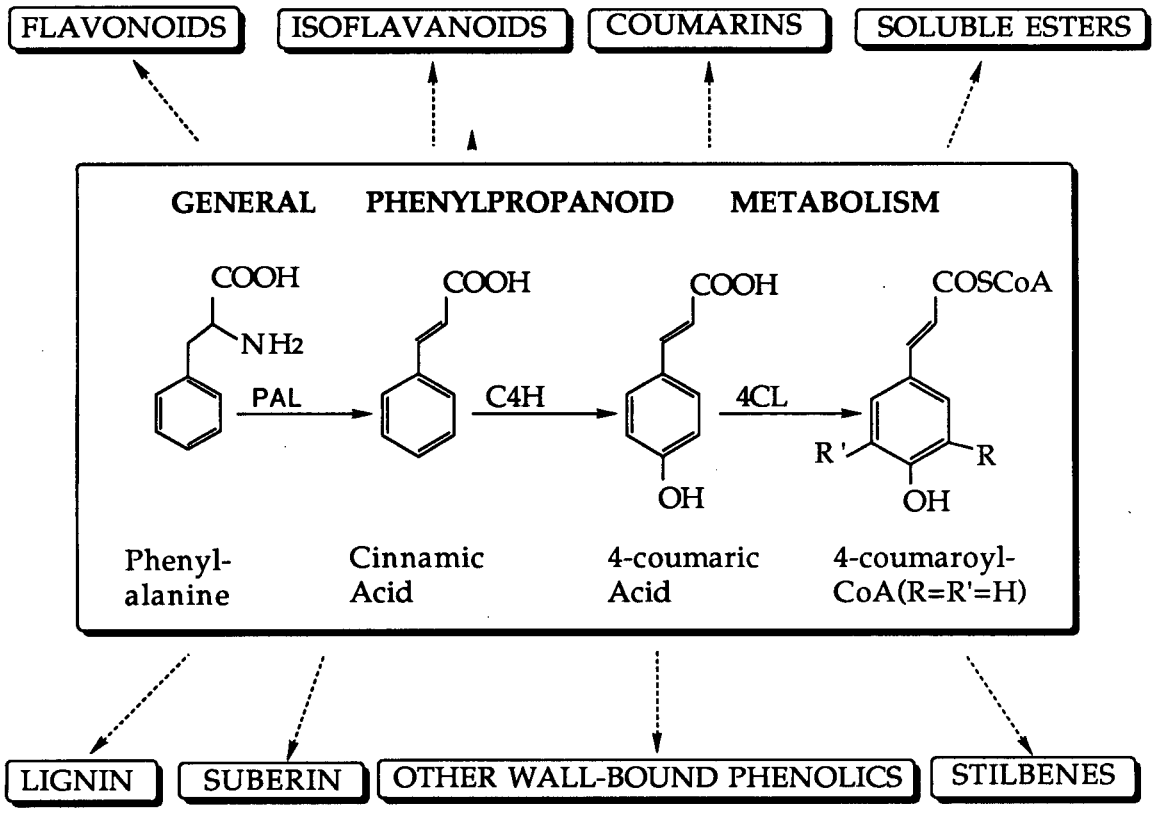


Figure 1. Schematic showing core reactions of general phenylpropanoid metabolism as well as some of the major branch pathways.

microbial invasion. The hydroxycinnamic acids are known to be covalently bound to lignin, suberin, and the matrix polysaccharides .

The induction of lignification as a defence response has been observed in many plants (Grisebach, 1981; Hahlbrock and Grisebach, 1979; Vance et al., 1980). Several modes of action have been proposed (Vance et al, 1980). Lignification could enhance the resistance of plant cell walls against mechanical or enzymic attack and thus impede fungal invasion in host cells. Incrustation of cell walls with lignin may also slow down diffusion and thus immobilize fungal toxins or decrease the flow of nutrients to the pathogen. The phenolic precursors as well as the free radicals formed during the process of lignification may be fungitoxic and act as phytoalexins.

Lignin is synthesized from hydroxylated and methoxylated cinnamyl alcohols synthesized in two steps from the Coenzyme A esters by cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase (CAD) (Grisebach, 1981). Elicitor-treated bean cell cultures show a transient increase in CAD enzyme activity (Hahlbrock and Scheel, 1989). Lignin accumulation has also been shown for castor bean cell cultures treated with a pectic fragment elicitor (Bruce and West, 1989). Wheat plants innoculated with an incompatible race of stem rust showed an increase in activities of PAL and 4CL, as well as CAD, which is specific to the lignin pathway, at the time of hypersensitive host cell death. Treatment of the tissue with a-aminooxy-ß-phenylpropionic acid (AOPP), an inhibitor of PAL, prior to inoculation inhibited lignin deposition and resulted in fungal invasion (Moerschbacher, 1989). Similar observations with other plants (Vance et al., 1980) support the idea that lignin formation is important in actively protecting the plant against pathogens.

The phytoalexin response is thought to be ubiquitous in plants (Dixon, 1986). There are over 200 known phytoalexins belonging to a variety of

structural classes some of which are derived from general phenylpropanoid metabolism (Dixon, 1986; Ebel, 1986). Phytoalexins have been isolated from over 20 plant families (Dixon, 1986), and have been shown to be toxic to fungi and bacteria (Dixon, 1986). The mechanisms by which phytoalexins achieve their toxic effects are unknown, but considering the great diversity of phytoalexin structures a single mode of action is very unlikely.

Phenylpropanoid-derived phytoalexins have been well characterized in parsley and bean (Hahlbrock and Scheel, 1989; Templeton and Lamb, 1988). These two plants have served as model systems in which to study the phytoalexin response in herbaceous plants. The putative phytoalexins of parsley, the furanocoumarins, have been shown to accumulate in cell cultures treated with the fungal elicitor Pmg, a cell wall preparation from *Phytophthora megasperma* f. sp. *glycinea* (Kombrink and Hahlbrock, 1985), as well as in parsley plants inoculated with the soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* (Jahnen and Hahlbrock, 1988). Furanocoumarin accumulation is preceded by transient increases in the mRNA levels of genes encoding the enzymes involved in general phenylpropanoid metabolism (PAL and 4CL) (Douglas et al, 1987), and enzymes specific to the furanocoumarin pathway (S-adenosyl-L-methionine:xanthotoxol O-methyltransferase (XMT) and S-adenosyl-L-methionine:bergaptol O-methyltransferase (BMT)) (Hauffe et al., 1986). *In situ* hybridization experiments with radiolabelled cDNA for BMT has shown that there is a significant increase in gene activity in the tissue surrounding the HR site, indicating that there is induction of phytoalexins (Hahlbrock and Scheel, 1989). Also, immunohistological studies have shown that there is a significant increase in the activity of the enzymes involved in general phenylpropanoid metabolism and furanocoumarin synthesis around the HR site and in oil-duct epithelial cells, where flavonoid and furanocoumarins

are synthesized at high rates (Jahnen and Hahlbrock, 1988). Furanocoumarin accumulation and high expression of the corresponding genes have also been reported in uninfected cotyledons where they seem to play a role in senescence (Knogge et al., 1987).

In bean (*Phaseolus vulgaris*), induction of isoflavonoid phytoalexins occurs in elicitor-treated cell cultures, in wounded hypocotyls, and in compatible or incompatible interactions with *Collitrichum lindemunthianum* (Lawton and Lamb, 1987), The response is much more rapid in the incompatible interactions, supporting the notion that the phytoalexin response is an important feature of disease resistance. Isoflavonoids are derived from a branch pathway of flavonoid biosynthesis, which is one of the branch pathways emanating from general phenylpropanoid metabolism. Prior to accumulation of isoflavonoid phytoalexins, there is a transient increase in the rate of transcription of PAL (Edwards et al., 1985; Lawton and Lamb, 1987), and genes specific to flavonoid biosynthesis, chalcone synthase (CHS) and chalcone isomerase (CHI) (Lawton and Lamb, 1987; Bell et al., 1984; Bell et al.,1986; Ryder et al., 1984; Mehdy and Lamb, 1987). Phytoalexin induction has been observed in other plants such as soybean (Ebel, 1986), potato (Hahlbrock and Scheel, 1989) pea (Ebel, 1986), and tobacco (Chapell et al., 1987), but in general the genes involved have not been well characterized as in parsley and bean.

Plant defence responses can also be triggered by elicitors from plant origin. PGA lyase (endopolygalacturonic acid lyase), an enzyme secreted by *Erwinia carotovora*, has been shown to elicit phytoalexin accumulation in soybean by releasing fragments from pectic polysaccharides in plant cell walls (Davis et al., 1984). In parsley cell cultures, plant cell wall fragments solubilized from parsley cell walls by PGA lyase have been shown to induce the accumulation of furanocoumarins (Davis and Hahlbrock, 1987). The plant cell

wall elicitor was found to act synergistically with the fungal glucan elicitor in the induction of furanocoumarins (Davis and Hahlbrock, 1987). This suggests that plant- and pathogen-derived compounds may act as elicitors during the interaction of potential pathogens with the intact plant. *Arabidopsis thaliana* cell cultures treated with crude preparations of PGA lyase also respond with increased enzyme activities for phenylpropanoid metabolism (Davis and Ausubel, 1989). Suspension cultures of castor bean respond to pectic fragment elicitor by turning on the biosynthesis of lignin (Bruce and West, 1989). Thus, plants have evolved a defence mechanism that responds to pectin-degrading enzymes in a way that does not depend on recognizing the enzyme molecules themselves. Instead, recognition is of pectic oligosaccharides released by partial depolymerization of pectic polymers present in the cell walls of plants. Such depolymerization seems to be required for infection by *Erwinia*. Therefore, endogenous elicitors function as regulatory molecules that initiate the synthesis and accumulation of phytoalexin and lignin in infected tissues. Thus, cell suspension cultures treated with elicitors might be a useful approach in understanding the nature of induced responses in poplar.

Most of the studies concerning plant defence responses have thus far focused on herbaceous plants. We hope to use poplar as a model system to study defence responses in woody perennials. Little or no information on induced defence responses such as phytoalexin and lignin synthesis has been obtained with poplar or even other woody perennials. Poplars have features which make them ideal for studying tree physiology and molecular biology. They are easily vegetatively propagated, have a small genome size (Parsons et al., 1989), and transformation by *Agrobacterium tumefaciens* and regeneration of transgenic plants is possible (Fillati et al., 1987).

Poplar is an economically important hardwood tree in North America and throughout the world. Its main use is in the pulp and paper industry. Production in short rotation-intensive culture (SRIC) plantations is limited by the leaf rust disease caused by *Melampsora*. Complete, hypersensitive resistance to Melampsora medusae is inherited as a single dominant gene in *Populus deltoides* (Eastern cottonwood), thus resistance to this disease is usually race-specific (Prakash and Heather, 1986). In *Populus trichocarpa* (Black cottonwood) resistance is quantitative in nature, ie, resistance is not race-specific (Hsiang and van der Kamp, 1985). Both rusts require two hosts to complete their life cycle. Their main alternate host is Douglas Fir, on which they undergo their sexual cycle. Rust-infected poplars undergo premature defoliation leading to drastic reductions in their growth rates (Shain and Miller, 1979). Poplar hybrids derived from *Populus trichocarpa x P. deltoides* crosses have been developed which exhibit superior phenotypes relative to the parents (Heilman and Stettler, 1985; Stettler et al., 1988). H11 is one of these hybrids. It grows especially fast and is apparently resistant to *Melampsora* rust.

The genus *Populus* is known to be rich in soluble phenolic compounds. Many derivatives of phenolic compounds have been isolated from the bark of *P. trichocarpa*, such as salicin, trichocarpin, salireposide, salicyl alcohol, cinnamic acid, p-coumaric acids, and others (Pearl and Darling, 1968; Pearl and Darling, 1971). Induction of phenolic glycosides as phytoalexins in freshly wounded stem sections of *P. tremuloides* when inoculated with mycelium of *Hypoxylum mammatum* and *Alternaria* have been observed (Flores and Hubbs, 1980). Also, increased levels of phenolics in leaf extracts 72 hours after mechanical damage has been observed (Baldwin and Schultz, 1983). In H11 leaves infected with *Melampsora* p-coumaric acid increased 6 to 8-fold (Jian, 1989). Enhanced levels of phenolic compounds have been observed around wound

sites in stems of *P. trichocarpa x P. deltoides* hybrids (Shain and Miller, 1982). Pinocembrin a 5,7-dihydroxyflavone, has been identified in expanding leaves and bud resin of *P. deltoides*, and has been shown to have antifungal activity against *Gladosporium cucumericum* and *Melampsora medusae* spore germination (Kemp and Burden, 1986). Thus, while little or no definitive work has been done on the nature of phytoalexins in poplar, induction of phenylpropanoid metabolism, as in other plants may play a role in defence against pathogen infection.

Another type of defence exhibited by plants is a wounding response. A systemic accumulation of specific mRNAs in response to wounding has also been observed in H11 hybrids. Two of these wound inducible genes, win6 and win8, have been sequenced and show a high degree of similarity to chitinases from bean, tobacco, and barley (Parsons et al., 1989).

The objective of this thesis is to use a poplar H11 hybrid cell suspension culture treated with elicitors as a simplified system to study inducible defence responses, especially as it pertains to phenylpropanoid metabolism, and also to identify potential defence metabolites. Cell suspension cultures offer several advantages over whole plants. They are easily propagated in synthetic media, occupy little growing space, and since they can be uniformly stimulated, localized responses are not a problem. In other systems, cell cultures have proved suitable for the elucidation of enzymology and induction mechanisms of phenylpropanoid metabolism.

MATERIALS AND METHODS

Cell Cultures

An H11 hybrid (*Populus trichocarpa x P. deltoides*) callus culture (Heilman and Stettler, 1985) derived from a crown gall tumor was kindly provided by T. Parsons and M. Gordon, University of Washington. Cell suspension cultures were initiated from fresh callus in liquid MS medium without hormones at pH 5.7 containing 0.3% sucrose and 1X vitamins (Sigma). Cell Suspension cultures were grown in 200 or 40 mls of medium in 1L or 250 ml Erlenmyer flasks at 25° C in the dark with continuous shaking at 125 rpm. These cultures were subcultured weekly by inoculating 5 or 30 mls of a 7-day old culture into 35 or 200 mls of fresh MS media.

Treatment of Cells with Elicitors

Cell suspension cultures were elicited by adding aseptic elicitor in aqueous solution. Cultures were returned to standard growth conditions immediately after addition of elicitor for the remainder of the experiment . Cells were harvested by vacuum filtration on 11 cm Whatman 1 filter paper, weighed and frozen in liquid nitrogen.

Elicitor Preparation

Polygalacturonic acid lyase (PGA lyase) crude and purified extracts were derived from culture filtrates of *Erwinia carotovora* grown on pectin containing medium as described (Davis et al., 1984). *Erwinia carotovora* was kindly provided by Dr. Davis. PGA lyase activity was measured as described (Davis et al., 1984) by the change in absorbance at 235 nm with polygalacturonic acid grade III as the substrate. Reaction mixtures contained 0.25% substrate (w/v), 50 mM TRIS-HCl and 1mM $CaCl_2$ at pH 8.5 in a total volume of 3.0 mls. One unit of PGA lyase activity was the amount required to

release 1 µmol unsaturated products/min at 30°C. This is equivalent to a change in the absorbance at 235 nm of 2.6.

Phytophthora megasperma f.sp. *glycinea* was grown as described by Kombrink and Hahlbrock (1986). A local strain of the pathogenic fungus *Fusarium oxysporum* (Fo) isolated from Douglas fir roots (not pathogenic on poplar hybrid H11-11 roots) was cultured on potato sucrose agar. Pmg and Fo elicitors were prepared from mycelial cell walls using the method of Ayers et al., (1976).

Enzyme Assays

Cell extracts used for all enzyme assays were prepared by grinding frozen cells in liquid nitrogen and sand, and further grinding in 200 mM TRIS-HCl pH 7.8 and 14 mM 2-mercaptoethanol. Homogenates were then mixed with 1/10 (w/v) Dowex 1X2 (Biorad) at 4°C for 20 min, then centrifuged for 15 min at 17,000 rpm and supernatants saved. Protein concentrations of cell extracts were determined by the Bradford Assay (Biorad).

PAL activity was detected spectrophotometrically by the change in absorbance at 290 nm using L-phenylalanine (Sigma) as a substrate as described (Ragg et al.,1981). Reaction mixture contained 1/10 vol enzyme extract, 10 mM phenylalanine, 110 mM TRIS, and 1.4 mM 2-mercaptoethanol. Enzyme activity was determined by using a molar extinction coefficient for cinnamic acid of 10,000 L/mol x cm.

CAD activity was determined spectrophotometrically as a change in absorbance at 400 nm using coniferyl alcohol as a substrate as described (Sarni et al.,1984). Reaction mixture contained 500 µM coniferyl alcohol, 200 µM $NaDP^+$, 890 µM TRIS-HCl pH8.8 and 1/20 vol of enzyme extract in a total volume of 1 ml. The molar extinction coefficient of coniferaldehyde used to

calculate CAD activity was 21,000 L/mole x cm as determined by Wyrambik and Grisebach (1975).

CHS activity was determined radiometrically as described by Schroder, Heller and Hahlbrock (1979) using 2-[^{14}C]-malonyl-CoA (Amersham CFA.570) and p-coumaryl-CoA (a generous gift from W. Heller, Gesellschaft für Strahlen- und Umweltforschung, Neuherberg, FRG) as substrates. The reaction mixture contained 10 µM p-coumaryl CoA, 10 µM [2-^{14}C]-malonyl-CoA, 100 mM KPO_4, 1.85 mM 2-mercaptoethanol, and 1/5 vol enzyme extract. The reaction product naringinine was extracted with 200 µl ethyl acetate. 50 µl of extract was dried in a scintilation vial, toluene added and the radioactivity measured in a liquid scintillation counter.

The activity of 1,3-ß-glucanase was assayed by measuring the rate of reducing sugar production with laminarin (Sigma) as the substrate using the procedure described by Kauffmann et al., (1987).

RNA Isolation and Northern Blots

Frozen tissue-cultured cells were ground to a fine powder in liquid nitrogen, total RNA extracted by the method of Parsons et al., 1989, and poly(A)$^+$ RNA isolated by oligo(dT)-cellulose affinity chromatography (Maniatis et al., 1982). RNA was separated on formaldehyde gels and blotted to Hybond (Amersham) membranes according to the manufacturers specifications. Plasmids were purified by standard methods (Maniatis et al., 1982). Radioactive hybridization probes were prepared using a random primed labelling kit (Boehringer). A 280-bp AvaII-BamHI fragment was isolated from a cDNA clone of the parsley 4CL-1 gene (Douglas et al., 1987; Lozoya et al., 1988) for use as a 4CL hybridization probe; a 1.5-kb potato PAL cDNA (Fritzemeier et al., 1987) was used as a PAL hybridization probe; a bean CHS cDNA clone (Ryder et al., 1984) was used as a CHS hybridization probe; and parsley cDNAs from four

elicitor-induced genes, Eli5, Eli7, Eli9, and Eli11, were also used as hybridization probes (Somssich et al., 1989). Hybridization conditions were as described (Douglas et al., 1987); low stringency washes were performed in 2X SSC at 65°C.

HPLC Analysis of Cell Extracts and Culture Filtrates

Extracts of leaf or tissue-cultured cells were prepared by the addition of 1.0 mL of 50% methanol to 0.5 g cells and incubation at 66°C for 1 h. Extracts were clarified by centrifugation and supernatants used for HPLC analysis. Triplicate extracts were prepared at each sampling and gave nearly identical results. Culture filtrates were acidified to pH 2, sequentially extracted with ether, ethylacetate, and butanol, taken to dryness, and redissolved in 1/50 volume 50% methanol before HPLC analysis. HPLC chromatography was performed by applying samples to an Alltech C8-MOS column (4.6 mm i.d.X 250 mm, 5 µm beads) and eluting with acetonitrile. Absorbance was monitored at 330 nm.

Thioglycolic acid extraction of cell walls

Methanol-washed cell walls were extracted with thioglycolic acid, redissolved in alkali, and the relative amounts of phenolic material quantified by measuring absorption at 280 nm, as described by Bruce and West, (1989). The procedure was scaled down by extracting 0.5 g cells in 1.5 ml methanol, and using correspondingly less of the subsequent reagents.

RESULTS

As part of the initial characterization of the H11 suspension-cultured cells response to elicitor treatment, the effect of culture age on the response was tested. A crude PGA lyase preparation was used to inoculate cells of varying ages, and PAL activity was assayed in treated and untreated cells. PGA

lyase, a pectinase secreted by the soft rot pathogen *Erwinia carotovora*, has been shown to induce plant defence responses in other plant systems (Davis et al., 1984) by releasing pectic fragments from plants cell walls, and these fragments in turn act as endogenous elicitors. PAL activity was chosen as an indicator of induction of phenylpropanoid metabolism since its activity is required for the biosynthesis of all phenylpropanoid compounds.

As shown on Figure 2A, H11 suspension-cultured cells continued to grow for 17 days after subculturing. PAL activity in untreated cells remained constant throughout this time, but in elicitor-treated cells there was a strong induction of PAL activity (Figure 2B). Cells were most responsive to elicitor treatment 4 days after subculturing and showed decreasing sensitivity with increasing age. PAL activity of 11-day old cells treated with elicitor showed almost no induction compared to untreated cells. Consequently, all further experiments were performed 4 days after subculture.

To determine whether the induction of PAL activity in cells treated with a crude preparation of PGA lyase was in fact due to the enzymatic activity of PGA lyase, and not some other factor in the crude preparation, H11 cell cultures were treated with autoclaved (25 min) and nonautoclaved preparations of purified and crude PGA lyase and assayed for PAL activity. As shown in Figure 3, pure PGA lyase does cause an induction in PAL activity compared to untreated controls and autoclaved PGA lyase. However, the fact that crude preparations of PGA lyase show a much greater induction of PAL activity than pure PGA lyase, and that autoclaved crude preparations also cause induction of PAL activity suggests that induction of PAL activity can not be solely attributed to the enzymic activity of PGA lyase. There seems to be nonlabile factors in the crude preparation which can also induce PAL activity.

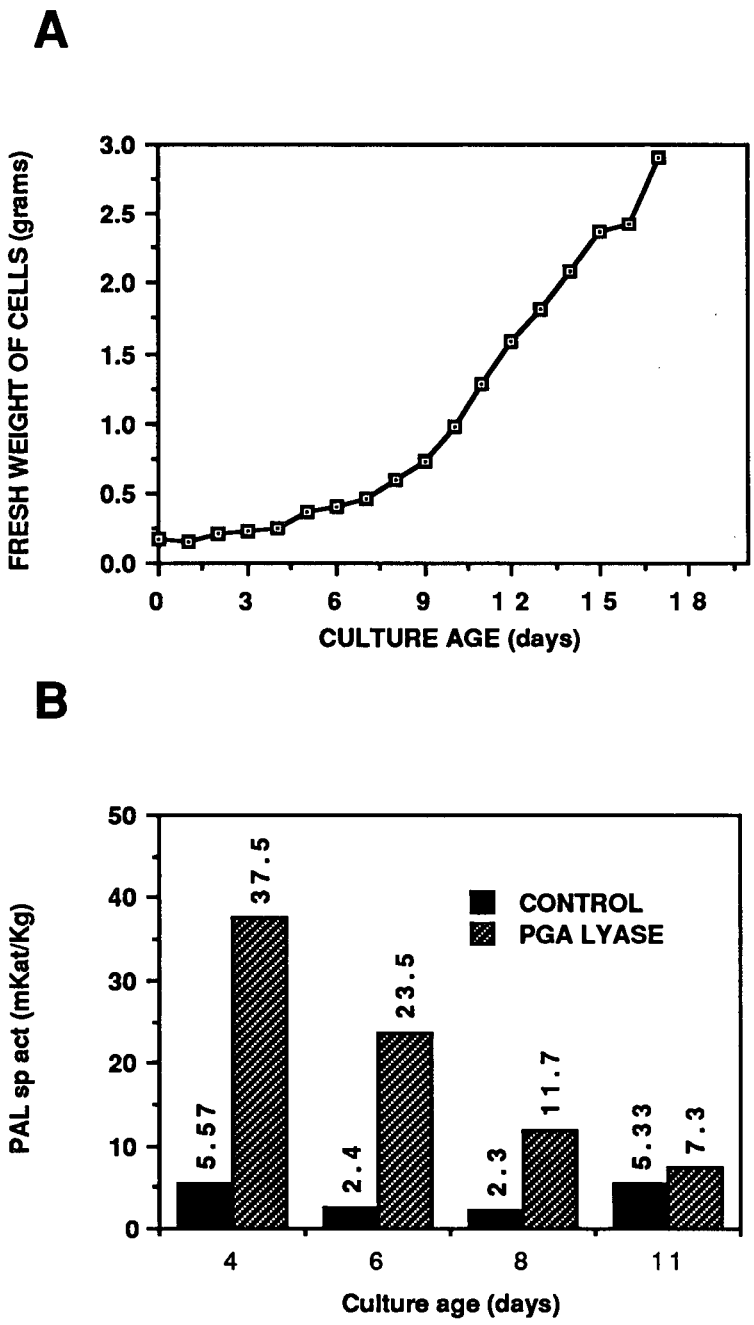


Figure 2. Poplar cell culture growth and elicitor responsiveness. (A) Growth curve of the poplar clone H11 suspension culture. Culture days refers to days after subculture. (B) PAL enzyme activity in untreated control cells and cells treated with 0.15 Units/ml PGA lyase at various times after subculture. Enzyme activity was measured 7 hr after elicitor application.

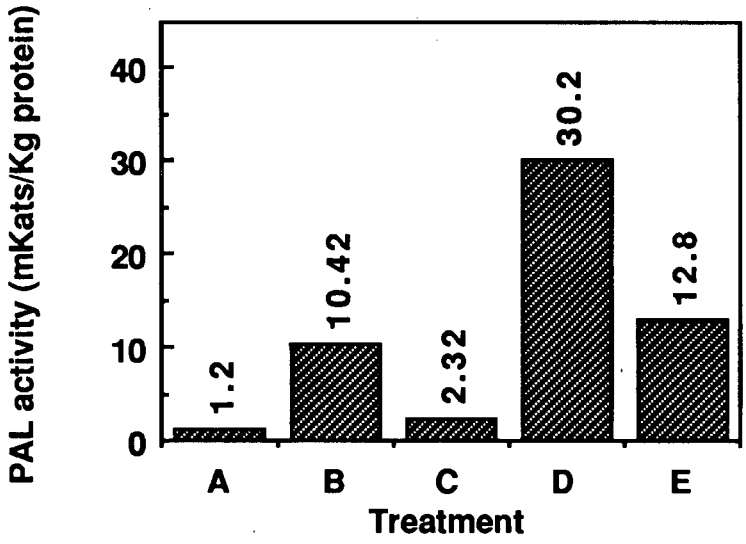
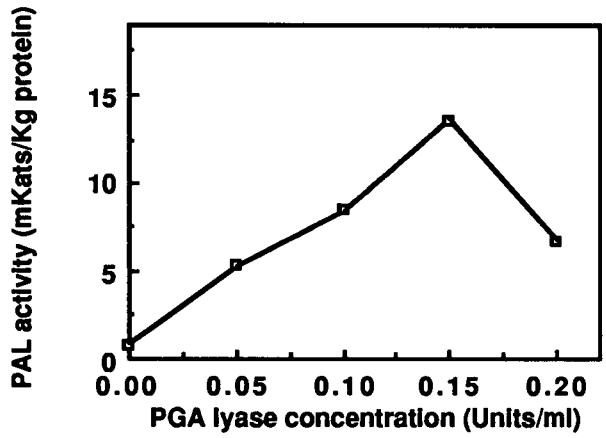


Figure 3. Differences in PAL enzyme induction in poplar cell cultures using purified PGA lyase (autoclaved and nonautoclaved) and PGA lyase crude extracts (autoclaved and nonautoclaved) as elicitors. (A) untreated, (B) treated with purified PGA lyase, (C) autoclaved purified PGA lyase, (D) crude extracts of PGA lyase, and (E) autoclaved crude extracts of PGA lyase. PAL enzyme activity was measured 7h after elicitor application to 4-day old cells.

Dose response curves of poplar cells to PGA lyase and two other elicitors were established to determine optimal concentrations for the induction of PAL activity (Fig. 4). In addition to a crude preparation of PGA lyase, an elicitor prepared from the cell walls of the soybean fungal pathogen *Phytophthora megasperma* f.sp. *glycinea*, (Pmg elicitor), which has been shown to induce defence reactions in other plants (Hahlbrock and Scheel, 1989; Kombrink and Hahlbrock, 1986), and a similar preparation from the cell walls of the fungus *Fusarium oxysporum*, a pathogen of Douglas fir and other conifers (Fo elicitor), were tested. PAL enzyme activity was assayed 7 hrs after elicitor application. In each case, PAL activity increased in a dose-dependent manner before reaching a maximum. The decrease in the ability of PGA to induce PAL activity at higher concentrations may be due to the loss of cell viability. In fact at these higher PGA lyase concentrations the fresh weight of cells was significantly reduced when compared with cells treated with lower PGA lyase concentrations (data not shown). Based on these results, further experiments were carried out using 0.15 Units/ml PGA lyase, 75 μg/ml Fo elicitor, and 100 μg/ml Pmg elicitor.

Kinetics of Phenylpropanoid biosynthetic enzyme induction

The kinetics of the induction of PAL activity in cells treated separately with the three elicitors was monitored over a period of 24 hrs following elicitor addition. As shown in Figure 5, there was a transient induction in PAL enzyme activity in cells treated with elicitor. Activities above basal level were clearly detectable by 3 hrs and reached maximum levels after 5-7 hrs after elicitor application, at which time levels were 10-20 fold above those in untreated control cells. By 24 hrs, activities had decreased almost to basal levels. While the kinetics of PAL induction were similar for the three elicitors, the level of inducible PAL enzyme activity varied. After 5-7 hrs, the cells routinely responded most strongly to the Fo elicitor, and least to the Pmg elicitor.



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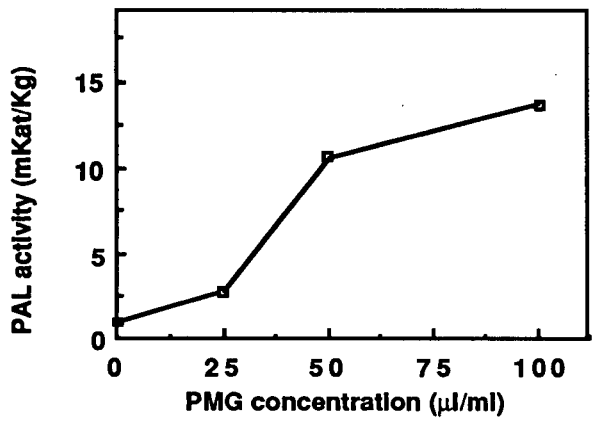

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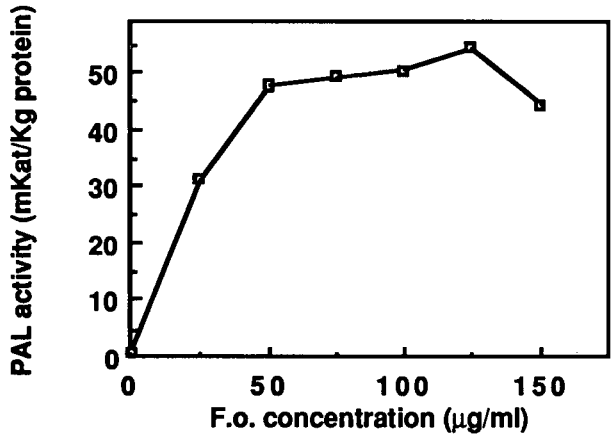


C

Figure 4. Dose response curves of suspension cultured cells to three elicitor preparations. (A) PGA lyase, (B) PMG, and (C) F.O.. PAL enzyme activity was measured 7 hrs after elicitor application to 4-day old cultures.

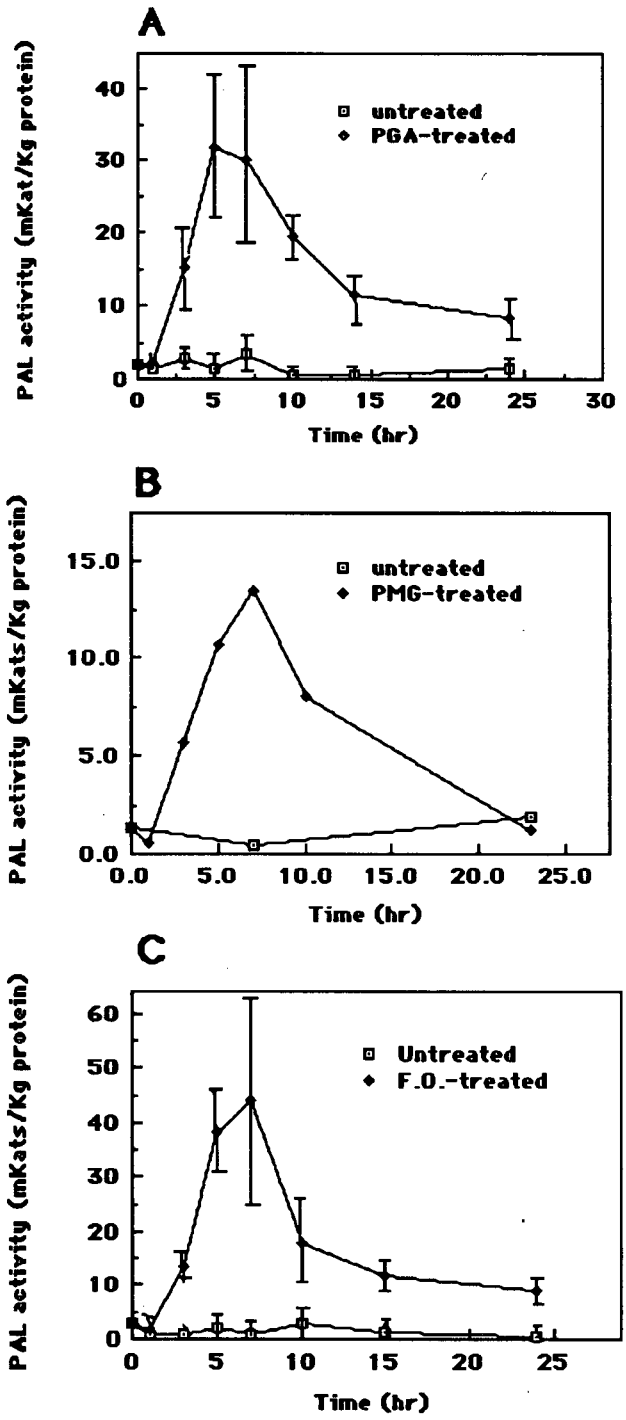
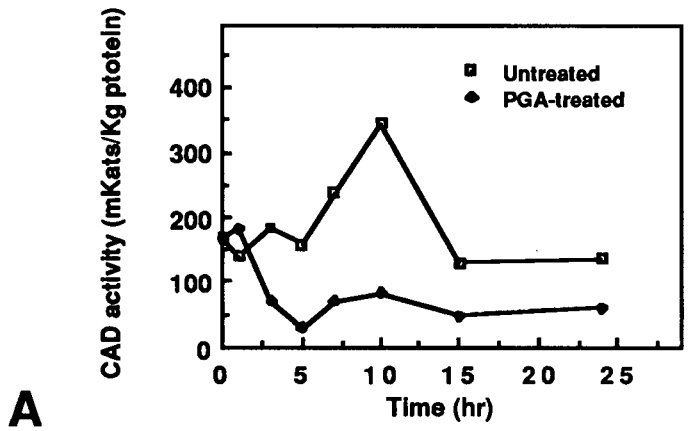


Figure 5. Changes in PAL enzyme activity in elicitor-treated and untreated control cell cultures. (A) PGA lyase-treated. (B) PMG-treated and, (C) FO-treated. Values in (A) and (C) are the mean + or - standard deviation of 4 separate experiments; in (B) they are the average of two separate experiments.



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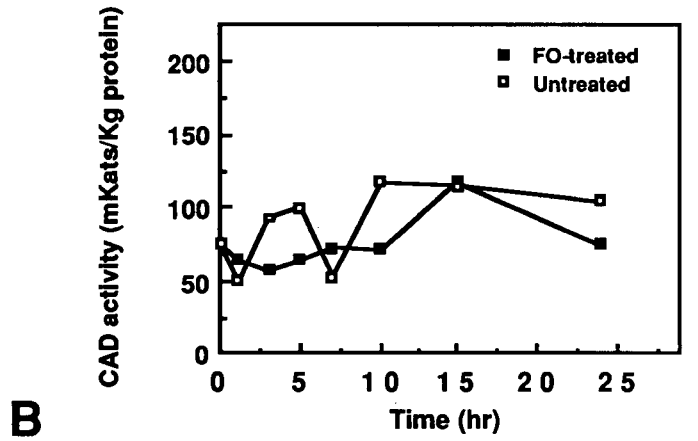

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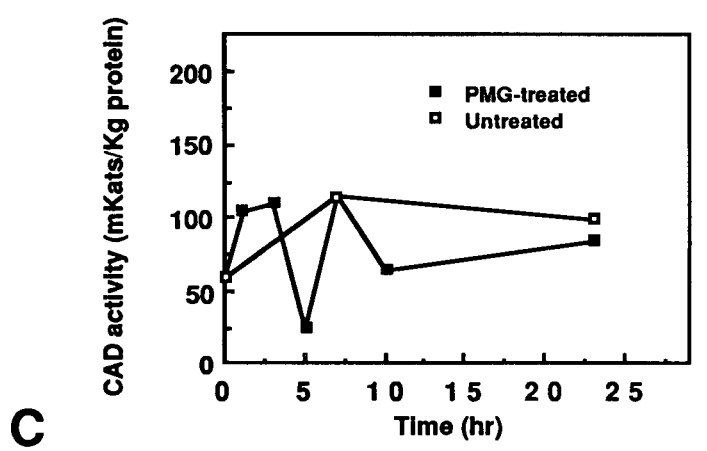

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Figure 6. Changes in CAD enzyme activity in elicitor-treated and untreated control cell cultures. (A) PGA lyase-treated, (B) Fo-treated and, (C) Pmg-treated. Values in (A) are the mean of two separate experiments, in (B) the mean of four separate experiments, and (C) one experiment.

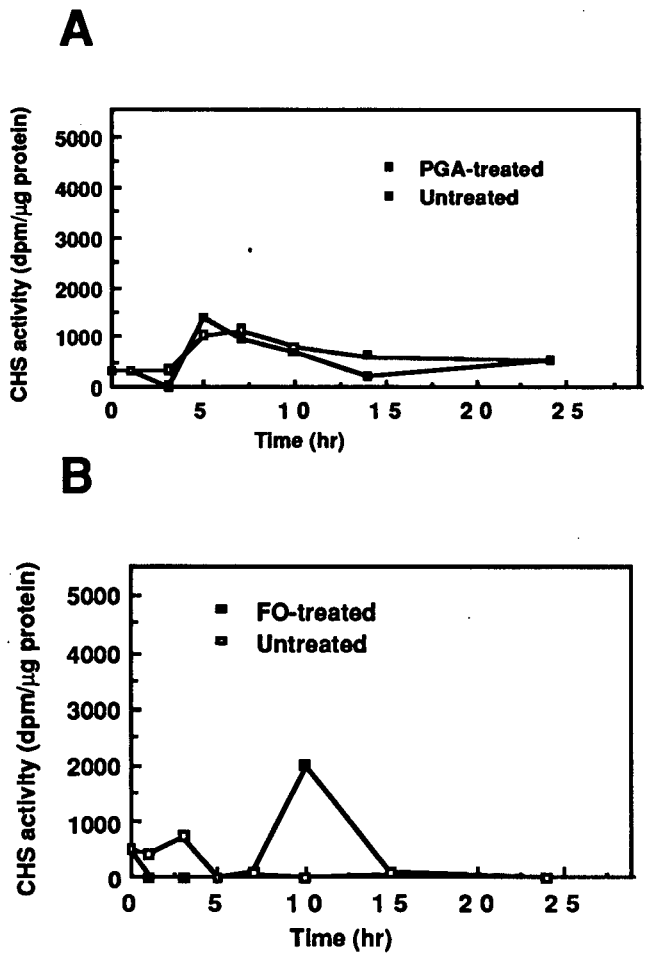

Figure 7. Changes in CHS enzyme activities in (A) PGA lyase-treated and untreated poplar cell cultures, and (B) FO-treated and untreated poplar cell cultures.

These results indicate that phenylpropanoid metabolism is rapidly induced in cells challenged with elicitors. Since the biochemical nature of the phenylpropanoid compounds being synthesized remained unknown, it was of interest to determine which phenylpropanoid branch pathways, if any, was activated in response to elicitor application. Unlike PAL, cinnamyl alcohol dehydrogenase (CAD), an enzyme specific for lignin biosynthesis, showed no detectable inducible activity above basal levels within 24 hrs of application of either PGA lyase, Pmg, or Fo elicitor (Figure 6). Similarly, chalcone synthase (CHS), an enzyme specific to flavonoid biosynthesis, showed no detectable inducible activity within 24 hrs after application of either PGA lyase, or Fo elicitor (Figure 7). These results suggest that activation of flavonoid and lignin biosynthetic enzymes may not be major components of the elicitor-induced defence mechanisms in these poplar cell cultures.

Kinetics of PAL, 4CL, and CHS mRNA Accumulation

It was of interest to find out whether the activation of PAL activity was preceded by an increase in PAL gene expression following elicitor treatment. It had previously been established that a potato PAL cDNA (Fritzemeier et al., 1987) cross-hybridized most efficiently with poplar genomic DNA on Southern blots (Douglas, Subramaniam, and Molitor, unpublished). This clone was used as a heterologous probe for hybridization to northern blots of poly (A^+) RNA isolated at various times from poplar cell cultures treated with PGA lyase. As shown in Figure 8A, there was a large and transient increase in the amount of an approximately 2.5-kb RNA hybridizing to the probe 1 hr after treatment with elicitor. This size is consistent with the size of PAL transcripts in parsley (Lois et al., 1989), potato (Fritzemeier et al., 1987), and *Arabadopsis*

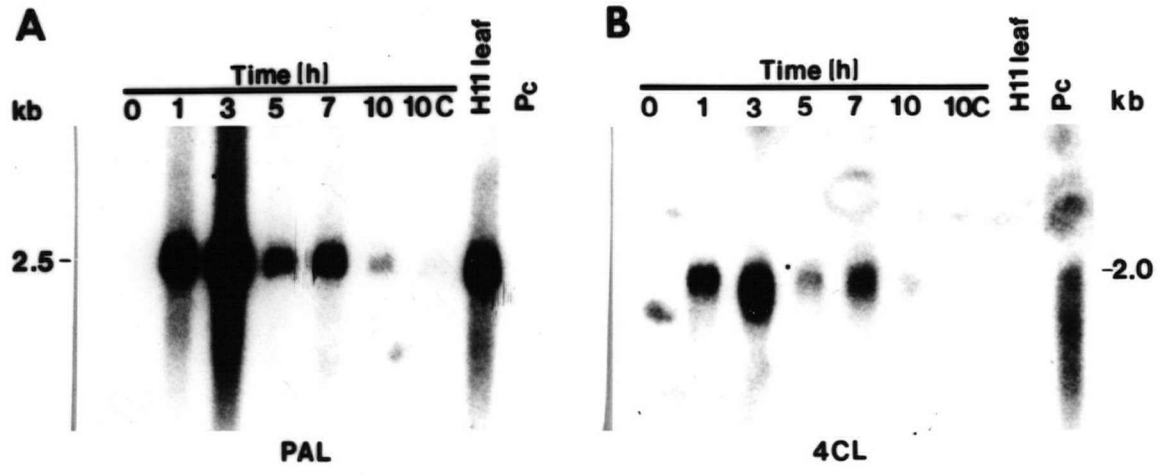

Figure 8. Time course of PAL and 4CL mRNA accumulation in cell cultures treated with 0.15 Units/ml PGA lyase crude extract. (A) PAL and (B) 4CL probes were hybridized to RNA blots of poly $(A)^+$ RNA (3 µg per lane) isolated from cells treated with elicitor for 1 to 10 hrs and from untreated cells at 0 and 10 hrs (10C). Poly $(A)^+$ RNA (3 µg) from young leaves of H11 poplar plants (H11) and 10 µg total RNA from Pmg-treated parsley suspension cultured cells (Pc) were also included. RNA sizes in kb were determined from migration of RNA standards.

(Davis and Ausubel, 1989). Maximum RNA levels were detected 3 hrs after elicitor treatment and declined to a level only slightly above background levels by 10 hours. To control for RNA amount on each lane, the same blot was subsequently hybridized with the cDNA of a constitutively expressed parsley gene, CON 2 (Somssich et al.,1989). RNA from each lane hybridized approximately equally to this probe, with the exception of the 5 hr lane, which appeared to be underloaded (data not shown). The potato PAL probe also hybridized to to a 2.5-kb RNA from young leaves of H11 poplar plants (H11 leaf, Figure 8A), in which PAL activities have been observed to be high (G. Subramaniam, F. Williams and C. Douglas, unpublished). In addition, there was strong hybridization to an identically-sized putative PAL RNA from elicitor-treated tobacco leaves (see Figure 10A, lane Nt). Lack of hybridization to RNA extracted from elicitor-treated parsley cells (Figure 8A, lane Pc) was expected, since the potato and parsley PAL genes do not cross hybridize (Fritzemeier et al., 1987). Similar results were obtained when a poplar cDNA fragment was used to probe the same blots (Fig. 9) indicating that the mRNA hybridizing to the probes is PAL mRNA.

In many plants, activation of 4CL gene expression occurs coordinately with that of PAL genes (Davis and Ausubel, 1989; Fritzemeier et al., 1987; Hahlbrock and Scheel, 1989; Lois et al., 1989). To detect poplar 4CL transcripts on northern blots, a 250-bp fragment of the parsley 4CL-1 cDNA (Lozoya et al., 1988) was used as a hybridization probe. This fragment lies within a region conserved between potato and parsley 4CL genes (Becker-Andre and Hahlbrock, 1991). The probe hybridized to an approximately 2.0 kb RNA in cells treated with PGA lyase, which accumulated with kinetics very similar to those for the putative poplar PAL RNA (Figure 8B). The RNA detected by the heterologous 4CL probe was of the same size as 4CL transcripts in elicitor-

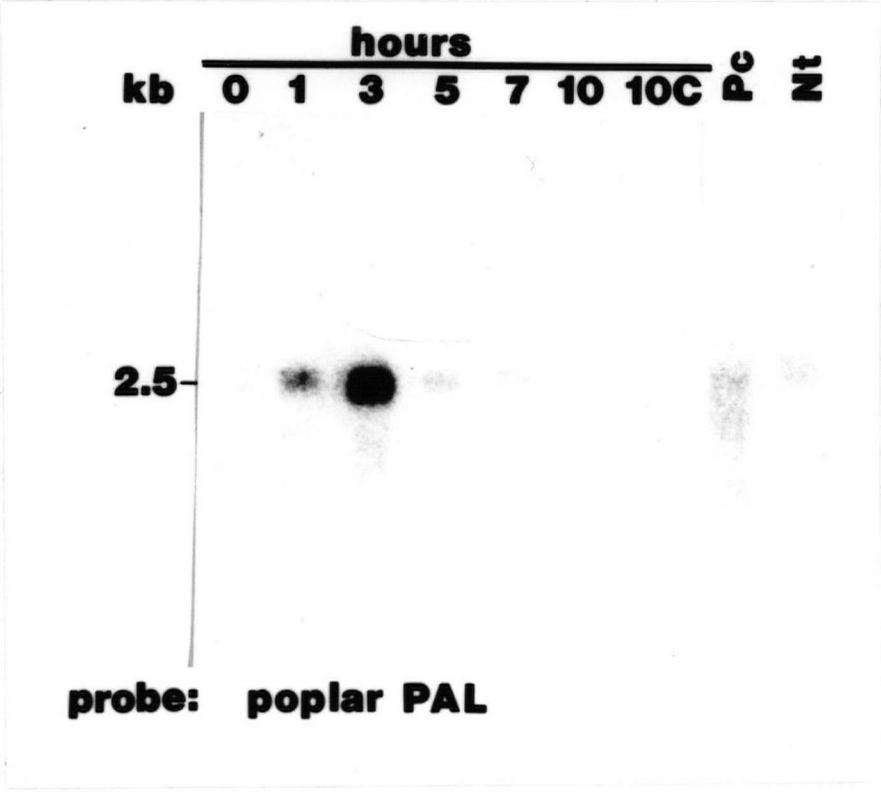

Figure 9. Time course of PAL mRNA accumulation in cell cultures treated with 0.15 Units/ml of PGA lyase crude extract. A poplar PAL probe was hybridized to RNA blots of poly $(A)^+$ RNA (3 µg per lane) isolated from elicitor-treated cells for 1 to 10 hrs and untreated cells 0 to 10 hrs (10C). Total RNA (10 µg) from Pmg-treated parsley cell cultures (Pc) was included; the Nt lane contains 3 µg poly $(A)^+$ RNA from Pmg-treated tobacco leaves. RNA sizes in kb were determined from migration of RNA standards.

treated parsley cells (Figure 8B, lane Pc) and the probe hybridized to a 2.0 kb RNA from H11 young leaf tissue and elicitor treated tobacco leaves (H11 leaf, Figs 8B and 10B; lane Nt, Fig. 10B). The weak hybridization to tobacco RNA was expected, since a full length parsley cDNA does not cross-hybridize to potato RNA (Fritzemeier et al., 1987). A control for RNA amount using the CON 2 probe was performed as described above, with similar results (not shown).

Since there was a large induction of PAL and 4CL mRNA accumulation 1 hr after elicitor treatment, earlier time points were analyzed to determine when detectable increases in mRNA amounts were evident. Figure 10 shows an increase in RNA hybridizing to the PAL probe 30 min after elicitor treatment, and an increase in 4CL mRNA amount was evident as early as 15-30 min after PGA lyase treatment. In addition, PAL and 4CL probes hybridized to mRNAs in elicitor-treated tobacco leaves (Nt lanes) which were identical in size to the putative poplar PAL and 4CL mRNAs. Hybridization of the same filters to the cDNA of a parsley gene whose expression is not affected by elicitor treatment showed that approximately equal amounts of RNA were loaded in each lane (the CON 2 probe hybridized weakly to tobacco and poplar leaf tissue). Therefore, accumulation of PAL and 4CL mRNAs in the poplar cells appears to accumulate almost immediately following elicitor application.

As a confirmation of the results showing that CHS activity was not induced in response to elicitor treatment, a bean CHS cDNA was used as a heterologous probe for hybridization to northern blots of poly(A^+) RNA isolated at various times from PGA lyase-treated poplar cell cultures. No hybridization was observed indicating that either CHS gene expression is not activated in response to PGA lyase, or that the bean CHS does not cross-hybridize with the poplar CHS.

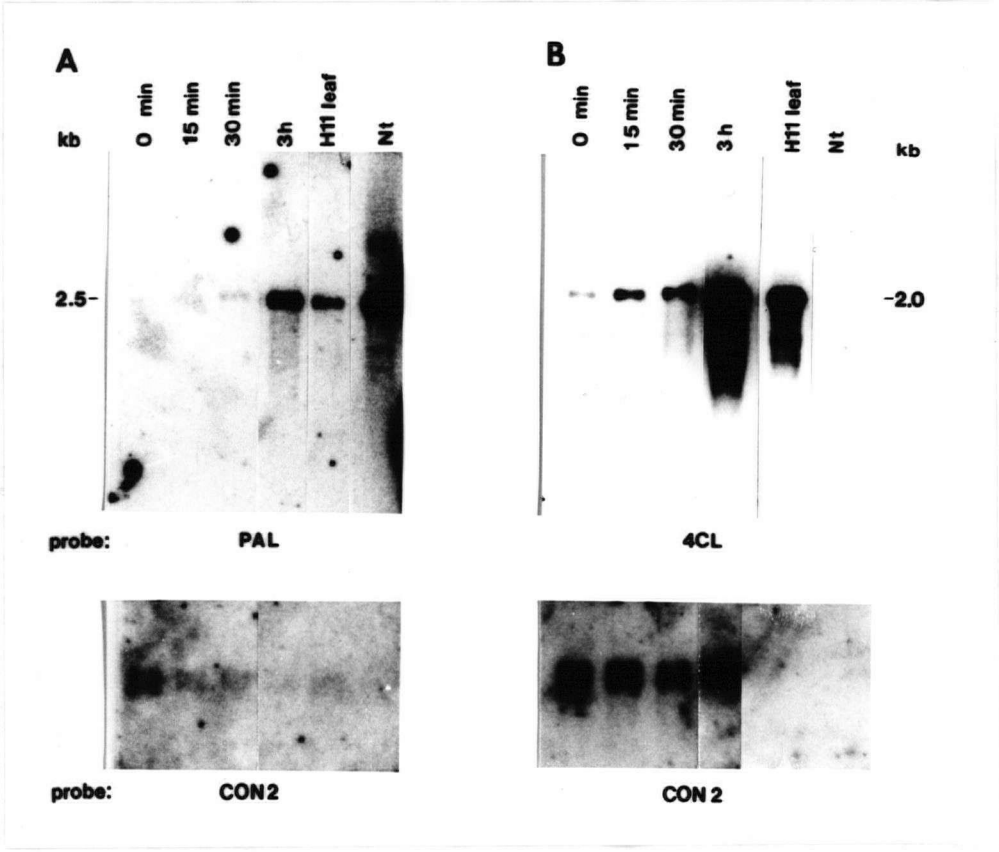

Figure 10. Rapid accumulation of PAL and 4CL mRNAs in cell cultures treated with 0.15 Units/ml of PGA lyase crude extract. RNA blots of poly $(A)^+$ RNA (3 µg per lane) isolated from elicitor-treated cells for 0 min to 3 hrs were hybridized to (A) PAL and (B) 4CL probes. The same blots were stripped and rehybridized to CON 2, a parsley gene unaffected by elicitor treatment. H11 leaf is as in Fig. 8; the Nt lane contains 3 µg poly $(A)^+$ RNA from Pmg-treated tobacco leaves. RNA sizes in kb were determined from migration of RNA standards.

Hybridization With cDNAs From Parsley Elicitor-Induced Genes

Recently, Somssich et al. (1989) have cloned a large number of cDNAs corresponding to genes that are rapidly activated upon treatment of cultured parsley cells with fungal elicitor. It was of interest to determine if there were corresponding genes in poplar cell culture which were also elicitor inducible. Four of these elicitor-induced genes, all having differing kinetics of mRNA accumulation in parsley, were used as heterologous probes to screen northern blots of poly(A^+) RNA isolated at various times from poplar cell cultures treated with PGA lyase. No hybridization was detected with three of the probes (Eli5, Eli7 and Eli9), indicating that either the corresponding genes are not activated in response to PGA lyase. Alternatively, these probes may not cross-hybridize with any poplar genes. However, hybridization was observed in lanes containing poly(A^+) mRNA from untreated and PGA lyase-treated cells when Eli 11 was used as the probe (data not shown). In addition, hybridization was more pronounced in the lane containing poly(A^+) mRNA from cells treated with PGA lyase for 3 hrs, suggesting that it may be elicitor-inducible or that this lane was over-loaded. Clearly more work is needed to positively conclude that this gene is elicitor-induced in poplar cell culture.

Testing For ß-1,3-Glucanase activity

In many plants, ß-1,3-glucanase activity is induced in response to elicitor treatment (Kombrink et al., 1986). It was of interest to determine if this hydrolytic enzyme was also elicitor-induced in H11 poplar cell culture. As shown in Fig. 11, ß-1,3-glucanase activity in Fo-treated cells does not differ greatly from that in control cells. However, the activity of this enzyme does increase 3 to 4-fold in both treated and untreated cells during a 25 hr time course, and there may be a small induction in activity in treated cells relative to control cells at later time points.

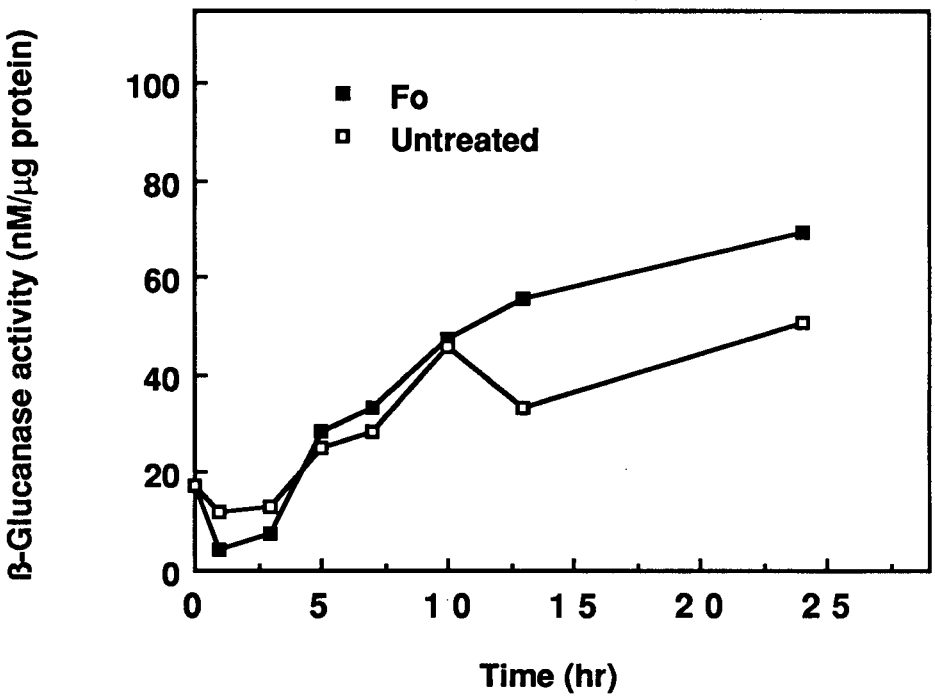

Figure 11. Changes in ß-1,3-glucanase activity in Fo-treated and untreated H11 hybrid poplar cell suspension cultures.

Phenolic Compound Accumulation

The above results indicate that H11 cell suspension cells treated with elicitors respond by activating general phenylpropanoid metabolism. The nature of the accumulating phenolic compounds, however, remained unknown. Since in other systems lignin accumulates in response to elicitor application to cell cultures (Bruce and West, 1989) or in pathogen infected tissue (Moerschbacher, 1989), it was of interest to determine if there was an increase in cell wall-bound phenolics in elicitor-treated cells. This was determined using the thioglycolate assay, which is specific for lignin and "lignin-like" compounds such as suberin (Bruce and West, 1989). To test for the presence of suberin, cells were stained with Sudan black B. No differences were observed in the intensity of staining between elicitor-treated and nontreated cells indicating that suberin biosynthesis is not induced in response to elicitor treatment (data not shown). As shown in Figure 12, H11 suspension cells treated with Fo elicitor responded with an increase in the rate of deposition of thioglycolic acid-extractable phenolic material as compared to untreated cells. An increase above basal level in the amount of this material was first detected 10 hr after elicitor application, and reached a maximum after 30 hr, at which time levels were more than two fold above those in untreated control cells. This level remained constant up to 40 hr.

To determine if there was an accumulation of soluble phenolic compounds in cells after activation of general phenylpropanoid metabolism in elicitor-treated cultures, cell extracts and culture filtrates were subjected to HPLC chromatography. The column was eluted by increasing the acetonitrile concentration from 0 to 20% for the first 10 min, then maintained at 20% until 25 min, and then increased gradually until 35 min at which time it was at 100%. In

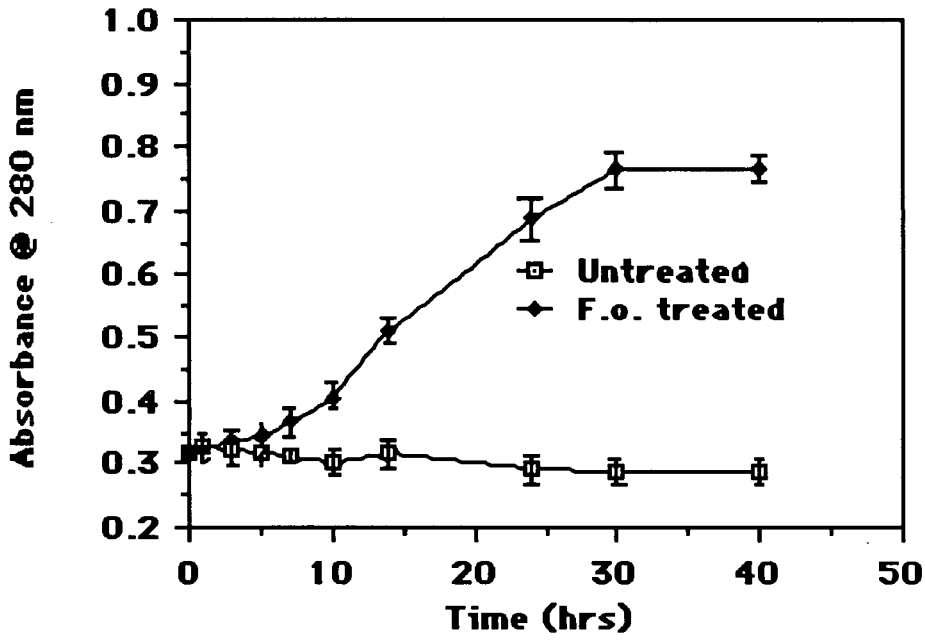

Figure 12. Time course of biosynthesis of thioglycolic acid-extractable cell wall phenolic compounds in elicitor-treated and non-treated poplar cell suspension cultures. Cells were 4-days old at the time of F.O. elicitor addition. Thioglycolic acid-extractable cell wall phenolic compounds were measured from 0.5 g of fresh weight of cells

contrast to HPLC profiles of methanol extracts from H11 leaves, which show a complicated mixture of compounds (Douglas and Williams, unpublished), relatively simple profiles were obtained with methanol extracts of cultured cells (Fig. 13, 14, and 15).

HPLC profiles of methanol extracts of cells from untreated cultures are shown on Fig. 13. Cells from these control cultures showed two major peaks eluting at 21.5 and 24 min. The HPLC profiles of methanol extracts of PGA lyase-treated cells and Fo-treated cells at various times after elicitor treatment are shown in Figures 14 and 15, respectively. Extracts of cells treated with elicitor consistently accumulated a compound eluting at about 24 min to approximately 3-fold relative to the same product in untreated cells (Fig. 13) and relative to a compound eluting 21.5 min in treated and untreated cells. Maximum accumulation of this compound was about 24 hr after elicitor addition. Consistent with the stronger induction of PAL activity by the Fo elicitor compared to PGA lyase, Fo-treated cells accumulated higher amounts of this compound. The three peaks eluting before 5 min are peaks associated with the solvent.

To test for the presence of phenolic compounds secreted by the cultured cells, culture filtrates were acidified to pH 2 and sequentially extracted with ether, ethylacetate, and butanol (ie. progressively polar solvents). This protocol should partition any compounds in the filtrates by their charge, such that nonpolar compounds are extracted with ether, and the most polar compounds are extracted with butanol. The samples were then concentrated and redissolved in 50 % methanol. No ether-extractable compounds were detected in filtrates from control or elicitor-treated cells (data not shown). However,

there was an accumulation of ethylacetate-extractable compound or compounds eluting as a broad peak at 27 min in filtrates from cells treated 24 hr with Fo elicitor (Fig. 16B). This peak was absent in control cells (Fig. 16A). In addition, at least two butanol-extractable compounds eluting at approximately 28 min were observed in filtrates from cells treated 24 hr with Fo elicitor (Fig. 16D). These peaks were also absent in control cells (Fig 16C). Qualitatively similar results were obtained when PGA lyase was used as the elicitor, but lower amounts of phenolic products were accumulated (data not shown). These results are consistent with the lower level of PAL enzyme activity induced by PGA lyase as compared to Fo. The precise nature of these accumulating compounds remains unknown.

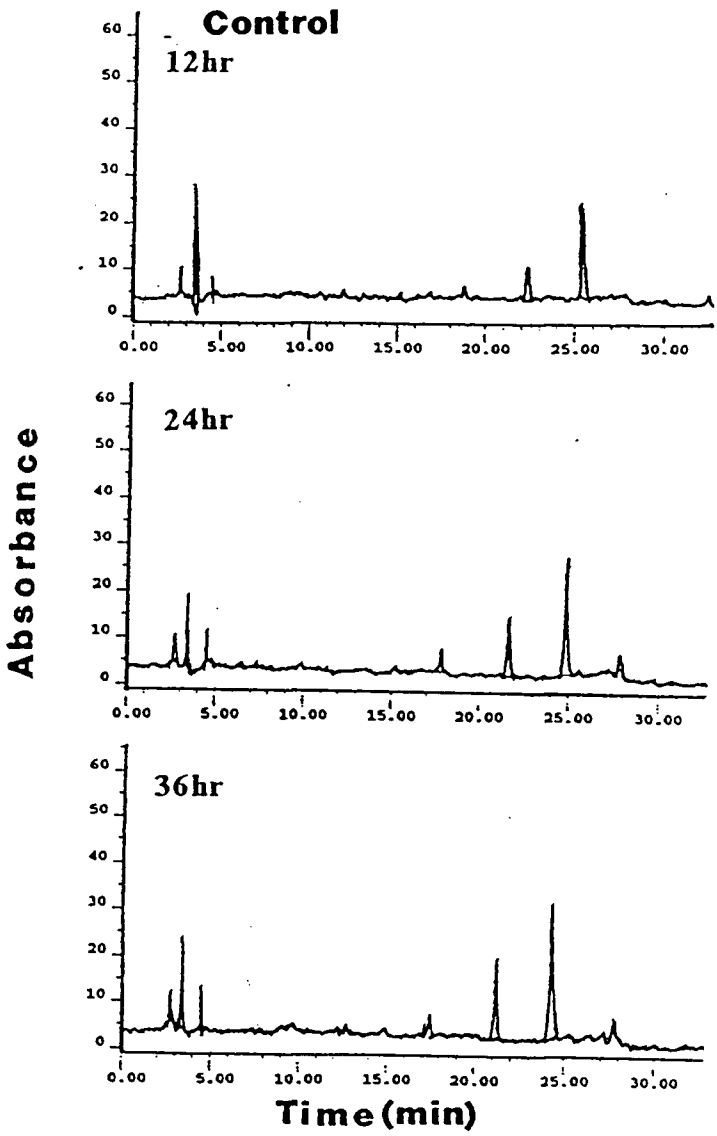

Figure 13. HPLC profile of methanol extracts of untreated H11 suspension-cultured cells. Untreated cells were harvested 12, 24 and 36 hrs after the start of elicitor treatment of separate cell cultures (shown on Figs. 14 and 15). Absorbance was read at 330 nm.

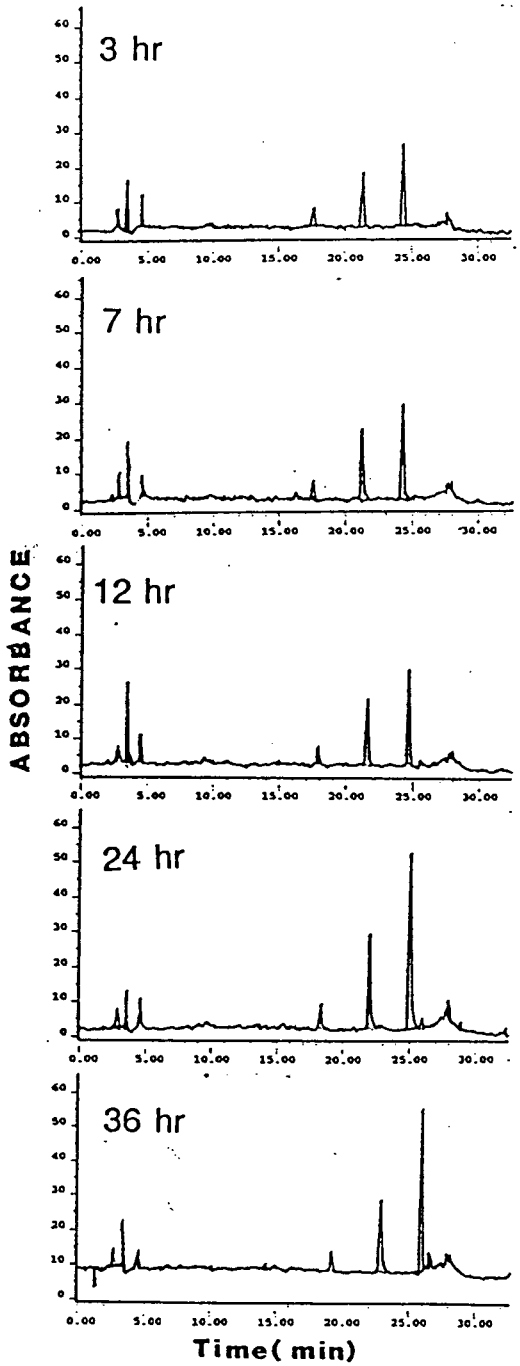

Figure 14. HPLC profile of methanol extracts of PGA lyase-treated cultured cells harvested at various times after elicitor treatment. Absorbance was read at 330 nm.

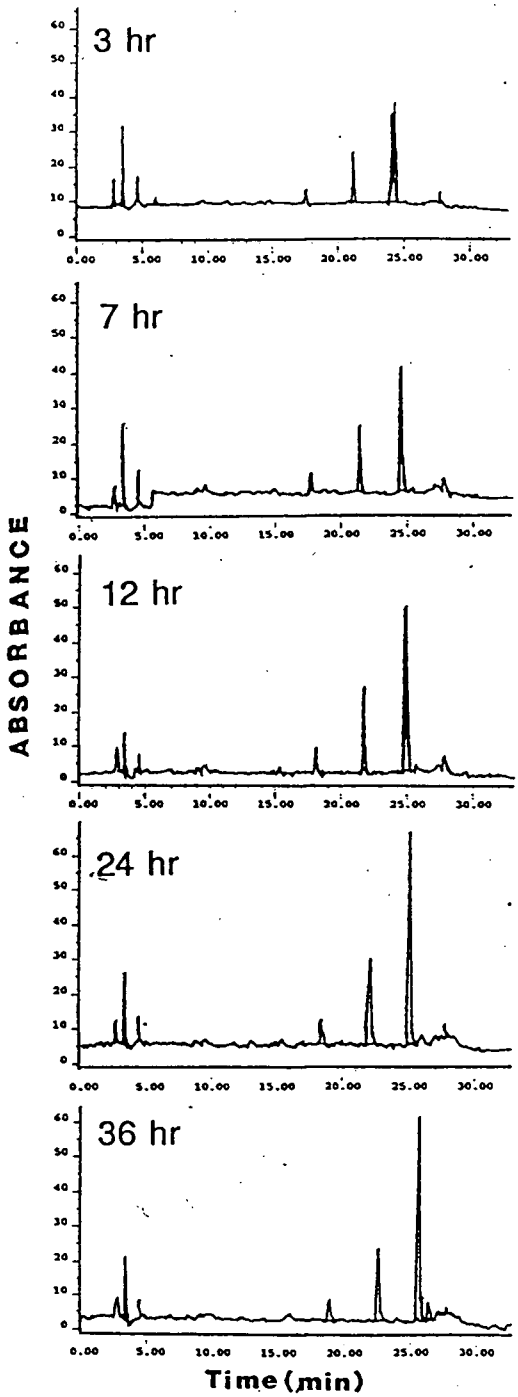

Figure 15. HPLC profile of methanol extracts of Fo-treated cultured cells harvested at various times after treatment. Absorbance was read at 330 nm.

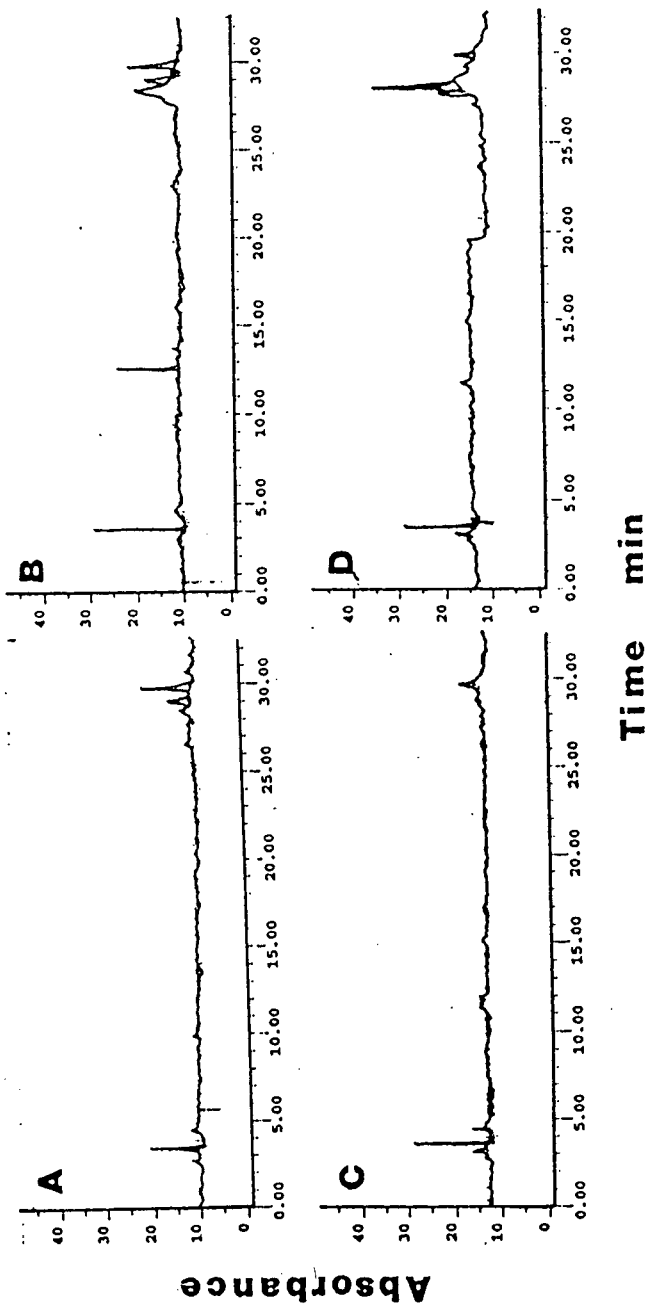

Figure 16. HPLC profiles of solvent extracts of culture filtrates from control and Fo-treated cells harvested 24 hrs after elicitor application. (A) Ethyl acetate-extracted filtrate from control cell culture. (B) Ethyl acetate-extracted filtrate from Fo-treated cell culture. (C) Butanol-extracted filtrate from control cell culture. (D) Butanol-extracted filtrate from Fo-treated cell culture.

DISCUSSION

This work has shown that suspension-cultured tumor cells from the fast-growing *Populus trichocarpa x P. deltoides* hybrid H11 respond to treatment with elicitors by rapidly activating phenylpropanoid metabolism. The activation proceeds sequentially from PAL and 4CL mRNA accumulation to a large increase in extractable activity of PAL enzyme and finally to the accumulation of specific phenolic metabolites in the cell walls, cell extracts and culture filtrates. This induction of phenylpropanoid metabolism is similar to the response observed in other plant cell cultures treated with elicitors (Dalkin et al., 1990; Davis and Ausubel, 1989; Hahlbrock Scheel, 1989; Lamb et al., 1989) as well as in pathogen-infected tissue of these plants (Fritzemeier et al., 1987; Hahlbrock and Scheel, 1989; Lamb et al., 1989; Jahnen and Hahlbrock, 1988; Schmelzer et al., 1989). Thus, as in these plants, phenylpropanoid metabolism appears to play an important role in poplar defence against pathogen infection.

It is noted that these H11 cultured cells are transformed with the T-DNA from a wild-type *Agrobacterium tumefaciens*. These transformed tumor cells differ from nontransformed cells in that they synthesize, accumulate and secrete opines. In addition, they also produce high levels of T-DNA encoded auxins and cytokinins which maintains them in an undifferentiated state. The fact that these cells are physiologically altered, however, does not detract from their usefulness in studying elicitor-induced responses.

Many features of phenylpropanoid metabolism induction in poplar cell cultures are similar to observations in other plant systems. The growth stage of poplar cell culture is important in determining the strength of response to elicitor-induction. These results are consistent with those observed in parsley cell cultures treated with elicitor (Kombrink and Hahlbrock, 1985). In addition,

poplar cells treated with three elicitors from diverse origin react qualitatively in the same manner. Similar results have been observed in parsley (Davis and Hahlbrock, 1987; Kombrink and Hahlbrock, 1986), alfalfa (Dalkin et al., 1990) and *Arabidopsis* (Davis and Ausubel, 1989). For example, in parsley, fungal elicitors from diverse origins all trigger the activation of the same group of furanocoumarin phytoalexins all showing similar kinetics of induction (Kombrink and Hahlbrock, 1986). PGA lyase preparations from the soft rot pathogen *Erwinia carotovora* are known to elicit defence responses by enzymatically releasing endogenous pectic fragments of plant origin which then act as elicitors (Davis et al., 1984). Thus, as in parsley (Davis and Hahlbrock, 1987), soybean (Davis et al., 1984), alfalfa (Dalkin et al., 1990), and *Arabidopsis* (Davis and Ausubel, 1989), poplar defence responses can be triggered by elicitors of fungal and endogenous plant cell wall origin.

In elicitor-treated poplar cells, extractable PAL enzyme activities were transiently elevated to levels 10- to 20-fold above those in untreated cells. In addition, basal levels of PAL activity in untreated cells were 50- to 100-fold higher compared to basal levels in *Arabidopsis* (Davis and Ausubel, 1989), parsley (Kombrink and Hahlbrock, 1986), and alfalfa (Dalkin et al., 1990). Similarly, CAD activities, albeit not inducible above basal levels in response to elicitor-treatment, are also higher in comparison to activities in alfalfa (Dalkin et al., 1990), but only 5-fold higher than basal activity in bean cells (Grand et al., 1987). One explanation for these high basal activities is that poplar cells are inherently more active in phenylpropanoid metabolism than cells of the herbaceous plants so far examined. This explanation has credibility since it is well documented that large numbers of phenolic compounds are synthesized in the genus *Populus* (Pearl and Darling, 1968 and 1971; Greenaway et al., 1990). Another explanation could be in the altered physiology of the

suspension-cultured cells. The H11 cell culture used in these experiments was derived from a stem crown gall tumor (T. Parsons and M. Gordon, personal communication), and grown in the absence of added phytohormones. Phytohormones levels can affect the levels of phenylpropanoid enzyme activities (Church and Galston, 1988; Hösel et al., 1982). Therefore, it is possible that this higher rate of phenylpropanoid metabolism in cultured cells is attributable to the relatively high levels of auxin and cytokinin produced endogenously from the expression of the *Agrobacterium tumefaciens* T-DNA genes.

The kinetics of accumulation of putative PAL and 4CL mRNAs were very similar to each other. This is comparable to other plants such as parsley (Grisebach, 1981; Hahlbrock and Scheel, 1989), *Arabidopsis* (Davis and Ausubel, 1989), and alfalfa (Dalkin et al., 1990) in which the activation of PAL and 4CL genes are highly coordinated in response to elicitor treatment of cells and/or fungal infection of leaf tissue. The rapid activation of PAL and 4CL gene expression in elicitor-treated cells is similar in timing to the elicitor activation of PAL mRNA accumulation and is consistent with the rapid increase in transcription of bean PAL and CHS genes in those cells after elicitor application (Templeton and Lamb, 1988).

We are very confident that the poplar mRNAs detected by heterologous probes PAL and 4CL do in fact represent the respective poplar genes. The RNAs detected were of the expected sizes and co-migrated with RNAs from elicitor-treated tobacco leaves, young poplar leaves, and elicitor-treated parsley cells which also hybridized to the probe. In addition, putative PAL and 4CL cDNA clones have been cloned from a poplar cDNA library which have high sequence similarities to the parsley and potato genes (G. Subramaniam, E. Molitor and C. Douglas, unpublished). Hybridization of these probes to northern

blots of RNA from elicitor-treated poplar cells yielded similar results to those obtained using the heterologous probes (Fig. 9).

The induction of ß-1,3-glucanase activity has been reported in tobacco leaves infected with TMV (Tobacco Mosaic Virus) (Kauffmann et al., 1987), elicitor-treated cell cultures (Kombrink et al., 1986), and during senescence of cotyledons (Knogge et al., 1987). In contrast to these systems, ß-1,3-glucanase was not highly inducible upon treatment with the Fo-elicitor, although the activity did increase over a period of 25 hr in treated and untreated cells. This increase in activity could be due to endogenous levels of auxin and cytokinin in the cell cultures due to the expression of the *Agrobacterium tumefaciens* T-DNA genes. It has been shown that ß-1,3-glucanase is regulated by auxin and cytokinin at the mRNA level in tobacco tissues (Shinshi et al., 1987).

A significant accumulation of thioglycolic acid-extractable wall-bound phenolic material was observed in elicitor-treated cells relative to levels in untreated cells. This increase was subsequent to the activation of PAL and 4CL expression. The thioglycolic acid assay has been reported by Bruce and West (1989) to be specific for lignin and lignin-like compounds such as suberin. However, since staining for suberin gave negative results it probably is not the substance accumulating in the cell cultures. A similar increase in thioglycolic acid-extractable phenolic material has also been observed in castor bean cells treated with elicitor (Bruce and West, 1989). Other than the apparent phenolic nature, there is no direct evidence with regards to the chemical composition of thioglycolic acid extracts. It is noted however that identification of cell wall-bound phenolic material as lignin is notoriously difficult. Further work is needed to identify this thioglycolic acid-extractable phenolic material as lignin.

The results show that cinnamyl alcohol dehydrogenase (CAD) is not induced in elicitor-treated cells prior to the accumulation of thioglycolic acid-

extractable material. It is known that CAD activity is needed for the synthesis of hydroxycinnamic acid lignin precursors (Lewis and Yamamoto, 1990; Vance et al., 1980; Grand et al., 1987). It is concluded that CAD activity is apparently not required for the increased deposition of these phenolic materials in the cell walls. This indicates that either the material being accumulated in response to elicitor treatment is not lignin, or that some other enzyme(s) subsequent to CAD in the biosynthetic pathway of lignin, such as glucosyl transferase, ß-glucosidase or peroxidase, are rate limiting and are elicitor induced. Glucosyl transferase activity is thought to be required for glycosylation and subsequent transport to the cell wall of monolignols (Lewis and Yamamoto, 1990). ß-glucosidase activity is thought to be required for hydrolysis of cinnamyl alcohol glucosides intermediates during the formation of lignin in the cell walls. Such a role for these enzymes has been implicated in spruce seedlings where a cell-wall-bound glucosidase capable of hydrolysing coniferin (a phenolpropanoid glucoside) has been found (Grisebach, 1981). Elicitor-induced peroxidases (Bruce and West, 1989) have also been implicated in the final polymerization of the monolignols by oxidizing the phenoxyl group of these monomers, yielding phenoxy radicals which combine to form the final lignin polymer (Grisebach, 1981).

The prevalence of phenolic compounds in the genus *Populus* is well established (Pearl and Darling, 1969 and 1971; Flores and Hubbs, 1980; Greenaway et al., 1990; Jian, 1989; Shain and Miller, 1982). The HPLC profiles of cultured cell extracts is relatively simple, indicating that the accumulation of these compounds is suppressed in the H11 cell culture. This suggests that the synthesis of these compounds might be developmentally regulated. The progressive accumulation of certain soluble phenolic metabolites in poplar cells after elicitor treatment was clearly evident. An accumulation of phenolic

metabolites was also induced by 24 hrs after elicitor treatment in culture filtrates. These accumulating compounds have not yet been identified. However, since the activity of CHS, an enzyme specific for flavonoid biosynthesis, is not activated in elicitor-treated poplar cells, and CHS mRNA accumulation was not detectable, it is concluded that these compounds are probably not flavonoids. The partitioning of culture filtrates yielded some information regarding the nature of compounds accumulating extracellularly. Acidification of culture filtrates enhanced the extraction of these compounds, indicating that they are acidic in nature. In addition, the partitioning of some of the metabolites into the butanol phase is suggestive of glycosides since they are known to be very polar (Harborne, 1973). Chromatography of authentic standards showed that various simple phenolic and phenylpropanoid compounds and their glycosides (eg. p-hydroxybenzoic acid, gentianic acid, and coniferin, the glucoside of coniferyl alcohol) eluded with similar retention times of those compounds whose synthesis was increased in elicitor-treated cells (data not shown). Further work is clearly needed to chemically characterize the metabolites accumulating intra- and extracellularly.

It is possible that the soluble compounds accumulating in the elicited poplar cell cultures are synthesized in healthy leaf or other tissues of the intact plant, where they could be chemically or physically sequestered, as has been proposed for parsley furanocoumarin phytoalexins (Hahlbrock Scheel, 1989). In parsley, the synthesis of furanocoumarins is rapidly induced in elicitor-treated cells or in leaves at the sites of attempted fungal infection (Hahlbrock Scheel, 1989; Jahnen and Hahlbrock, 1988; Schmelzer et al., 1989), but is also developmentally regulated, apparently occuring primarily in the oil ducts of healthy leaves (Knogge et al., 1987; Schmelzer et al., 1989). By analogy,

phenolic compounds whose synthesis is developmentally regulated in poplar could also serve as anti-microbial defence compounds.

This tissue culture system will be useful in further elucidating stress-regulated phenylpropanoid gene expression in poplar. Since plant defence reactions triggered by elicitor treatment of cell cultures appear to faithfully reflect reactions triggered by pathogen infection of intact tissues (Hahlbrock and Scheel, 1989; Jahnen Hahlbrock, 1988; Schmelzer et al., 1989), the system should help in determining the role of poplar phenolics or phenylpropanoid metabolites in defence against pathogen infection. Future work will involve the study of inducible defence responses in pathogen-infected leaves of poplar, and comparing these results to the cell culture system. Also, more work is clearly needed to identify the compounds accumulating in the cell extracts, culture filtrates, and thioglycolic acid-extractable phenolic material from cells treated with elicitor. ß-glucosidase and peroxidase assays will also be done to determine if there is induction of lignin biosynthesis in elicitor-treated cells.

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