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Rapid Activation of Phenylpropanoid Metabolism in Elicitor-Treated Hybrid Poplar (*Populus trichocarpa* Torr. & Gray × *Populus deltoides* Marsh) Suspension-Cultured Cells[1]

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ABSTRACT

Elicitor induction of phenylpropanoid metabolism was investigated in suspension-cultured cells of the fast-growing poplar hybrid (*Populus trichocarpa* Torr. & Gray × *Populus deltoides* Marsh) H11-11. Treatment of cells with polygalacturonic acid lyase or two fungal elicitors resulted in rapid and transient increases in extractable L-phenylalanine ammonia lyase and 4-coumarate:coenzyme A ligase enzyme activities. The substrate specificity of the inducible 4-coumarate:coenzyme A ligase enzyme activity appeared to differ from substrate specificity of 4-coumarate:coenzyme A ligase enzyme activity in untreated control cells. Large and transient increases in the accumulation of L-phenylalanine ammonia-lyase and 4-coumarate:coenzyme A ligase mRNAs preceded the increases in enzyme activities and were detectable by 30 minutes after the start of elicitor treatment. Chalcone synthase, cinnamyl alcohol dehydrogenase, and coniferin β-glucosidase enzyme activities were unaffected by the elicitors, but a large and transient increase in β-glucosidase activity capable of hydrolyzing 4-nitrophenyl-β-glucoside was observed. Subsequent to increases in L-phenylalanine ammonia-lyase and 4-coumarate:coenzyme A ligase enzyme activities, cell wall-bound thioglycolic acid-extractable compounds accumulated in elicitor-treated cultures, and these cells exhibited strong staining with phloroglucinol, suggesting the accumulation of wall-bound phenolic compounds.

Induced responses are important in plant defense against pathogen infection, and their biochemical nature has been investigated in detail in many plants. Among the responses observed in pathogen-challenged cells are the induced accumulation of antimicrobial phytoalexins, synthesis of lignin and other wall-bound phenolic compounds, and synthesis of proteins such as chitinases and wall-associated hydroxyproline-rich glycoproteins (reviewed in refs. 16 and 22). An apparently ubiquitous feature of plant responses to pathogen challenge is the activation of phenylpropanoid metabolism (16, 22), suggesting general defensive roles for phenylpropanoid compounds. Representative reactions of phenylpropanoid metabolism include the first and last reactions of its general pathway, catalyzed by PAL[3] and 4CL, respectively, and those catalyzed by CHS and cinnamyl alcohol dehydrogenase, which are specific to flavonoid and lignin biosynthetic pathways, respectively.

The molecular basis for the induction of plant defense responses has received much attention (16, 22). These studies have been aided by the use of suspension-cultured cells treated with elicitors, which are compounds of fungal or plant cell wall origin that trigger defense reactions (22). In French bean and parsley suspension-cultured cells, elicitor-induced transcriptional activation of phenylpropanoid genes is followed by the transient increases in mRNA amounts and enzyme activities and by accumulation of final products (*e.g.* phytoalexins). The defense reactions triggered in tissue culture cells treated with elicitors appear to accurately reflect at least some of the reactions that occur in plant cells surrounding the sites of pathogen infection or attempted pathogen infection in whole plants in these and other systems that have been investigated in detail (16, 19, 22).

Relatively little is known about mechanisms of disease resistance in woody perennials. Ability to withstand infection by pathogens is especially important for such plants, since they have life spans of decades or longer. Trees in plantations are exposed to infection by potential pathogens, and resulting potential for losses in yield, for much greater time spans than annual crops. Poplars have features that make them useful model plants for the study of tree physiology and molecular biology. They are easily vegetatively propagated, have a small genome size (*c* value = 0.7 pg; ref. 26), and transformation by *Agrobacterium tumefaciens* and regeneration of transgenic trees are possible (8). Interspecific hybrids of North American western black cottonwood (*Populus trichocarpa* Torr. & Gray) and eastern cottonwood (*Populus deltoides* Marsh) have been developed for efficient production of fiber and fuel (17). In initial molecular studies of defense reactions in the *P. trichocarpa* × *P. deltoides* hybrid H11-11, it was shown that mRNAs encoding chitinases and other proteins accumulate systemically in plants subjected to severe wounding (26).

The genus *Populus* is known to be rich in soluble phenolic compounds (15), and various phenolic and phenypropanoid-

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[3] Abbreviations: PAL, L-phenylalanine ammonia-lyase; 4CL, 4-coumarate:CoA ligase; kb, kilobase; bp, base pair; CHS, chalcone synthase; PGA lyase, α-1,4-endopolygalacturonic acid lyase; SSC, standard sodium citrate; Pmg, *Phytophthora megasperma* f.sp. *glycinea*; Fo, *Fusarium oxysporum*.

derived compounds have been characterized in leaves and leaf buds (14, 15, 27). A few reports have ascribed defense-related properties to some such compounds. Pinocembrin (5,7-dihydroxyflavone), present in bud exudates of several poplar species, including *P. deltoides* (15), was shown to have antifungal activity (31), and accumulation of unidentified antifungal phenolic glycosides was reported to be induced in *Populus tremuloides* leaves after infection with fungal pathogens (9).

We are interested in the role of induced defense responses in the resistance of poplars to pathogen infection and report here the use of a cell culture system for the investigation of the nature of such responses in *P. trichocarpa* × *P. deltoides* hybrid H11-11.

MATERIALS AND METHODS

Plant Cells, Elicitors, and Elicitor Treatments

A crown gall tumor-derived callus culture from *Populus trichocarpa* × *Populus deltoides* hybrid H11-11 (17) was kindly provided by T. Parsons and M. Gordon, University of Washington. An H11-11 suspension culture was initiated by placing callus in liquid Murashige and Skoog medium without hormones; cultures were maintained in darkness with continuous shaking (125 rpm) at 25°C. Cells were subcultured weekly by inoculating 5 or 30 mL of a 7-d-old culture into 35 or 200 mL of fresh Murashige and Skoog media, respectively.

Crude extracts of PGA lyase were derived from culture filtrates of *Erwinia carotovora* (3) grown on pectin-containing liquid medium (3) at 24°C until late log phase (A_{600} 1.5 to 2.0). The medium was clarified by centrifugation and dialyzed against 10 volumes of 1 mM $CaCl_2$, 5 mM Tris-HCl, pH 8.5, overnight at 4°C and filter sterilized. PGA lyase activity was measured with polygalacturonic acid grade III (Sigma) as a substrate (3); 1 unit of PGA lyase activity is the amount required to release 1 μmol unsaturated products/min at 30°C. In some cases, PGA lyase was partially purified by chromatography of crude extracts through a CM-Sephadex C-50 column as described (3). *Phytophthora megasperma* f.sp. *glycinea* (Pmg) was grown as described (21), and a local strain of the phytopathogenic fungus *Fusarium oxysporum* (Fo) isolated from Douglas fir roots (not pathogenic on poplar hybrid H11-11 roots) was cultured on potato sucrose agar or liquid medium. Pmg and Fo elicitors were prepared from mycelial cell walls as described (21).

Elicitor treatments were initiated by the addition of aqueous elicitor solutions to suspension-cultured cells, which were returned immediately to standard growth conditions. Cells were harvested by filtration, weighed, and frozen in liquid nitrogen.

Enzyme Assays

Cell extracts used for all enzyme assays were prepared by grinding frozen cells in liquid nitrogen and sand, followed by further homogenization at 4°C in 200 mM Tris-HCl, pH 7.8, and 14 mM 2-mercaptoethanol. Homogenates were then mixed with 1/10 (w/v) Dowex AG type 1 × 2 (Bio-Rad) at 4°C for 20 min and centrifuged for 15 min at 34,500*g*, and the supernatants were retained. Protein concentrations in cell extracts were determined by the Bradford assay (Bio-Rad).

PAL (28) and cinnamyl alcohol dehydrogenase (13) enzyme activities were assayed spectrophotometrically. 4CL enzyme activity was measured spectrophotometrically (28) with 4-coumarate as a substrate. At selected time points, activity was also measured with caffeate, ferulate, and sinapate as substrates. In these cases, the same extract was used with the different substrates in parallel; accumulation of products was measured by the absorption maxima and extinction coefficients of the appropriate CoA esters (33).

CHS activity was determined radiometrically as described by Schröder *et al.* (30), with 2-[^{14}C]malonyl-CoA (Amersham) and *p*-coumaryl CoA (a gift of W. Heller, Gesellschaft für Strahlen-und Umweltforschung, Neuherberg, Germany) as substrates. Coniferin β-glucosidase activity was measured as described by Hösel *et al.* (18), with coniferin as a substrate (gifts of B. Ellis and G. H. N. Towers, University of British Columbia, Vancouver, British Columbia, Canada). β-Glucosidase activity was also measured with 4-nitrophenyl-β-glucoside as a substrate (18).

cDNA Library Construction and Screening

A cDNA library containing 2 × 10^6 recombinants was constructed in the λ-ZAP II vector (Stratagene, San Diego, CA) according to the supplier's instructions, with poly(A^+) RNA isolated from young leaf tissue of hybrid H11-11. A portion of the library was screened with a 1.5-kb potato PAL cDNA (10) as a hybridization probe; washes were conducted at reduced stringency (2× SSC at 65°C). Several putative PAL cDNAs were subjected to sequence analysis with double stranded plasmid DNA as templates (29). Sequence analysis was performed with DNA Inspector IIe software (Textco, West Lebanon, NH).

RNA Isolation and Analysis

Frozen tissue culture cells or leaf material was ground to a fine powder in liquid nitrogen; total RNA was extracted by the method of Parsons *et al.* (26), and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose affinity chromatography (29). RNA was separated on formaldehyde gels and blotted to Hybond (Amersham) membranes according to the manufacturer's specifications. Before transfer to membranes, gels were stained with ethidium bromide to ensure evenness of loading in each lane. Examination of membranes and gels for ethidium bromide-stained RNA after blotting indicated that transfer was even and essentially quantitative. Plasmids were purified by standard methods (29). Radioactive hybridization probes were prepared with a random primed labeling kit (Boehringer). A 280-bp *Ava*II-*Bam*HI fragment was isolated from a cDNA clone of the parsley 4CL-1 gene (7) for use as a 4CL hybridization probe; a 2.4-kb poplar PAL cDNA identified in this study was used as a PAL hybridization probe. Hybridization conditions were as described by Douglas *et al.*

(7); low stringency (4CL hybridization) washes were performed in 2× SSC at 65°C; high stringency washes (PAL hybridization) in 0.2× SSC at 65°C.

Thioglycolic Acid Extraction of Cell Walls

Methanol-washed cell walls were extracted with thioglycolic acid and redissolved in alkali, and the relative amounts of phenolic material were quantified by measuring A_{280}, as described by Bruce and West (1). The procedure was scaled down by extracting 0.5-g cells in 1.5 mL methanol and using correspondingly less of the subsequent reagents.

Phloroglucinol Staining

Cells were stained with a HCl-acidified, 5% ethanolic solution of phloroglucinol (BDH Chemicals). Before staining, cells were either used directly, extracted with 50% or 100% methanol at 66°C for 1 h, or extracted with methanol and then hydrolyzed for 1 h at room temperature in 1 M NaOH.

RESULTS

Effects of Different Elicitors on Suspension-Cultured Cells

The *E. carotovora* pectin-degrading enzyme PGA lyase has been shown to elicit plant defense reactions by releasing pectic fragments from plant cell walls, which in turn act as endogenous elicitors (3). We used PGA lyase preparations to initially characterize the elicitor responsiveness of H11-11 suspension-cultured cells and first established the time after subculturing of maximum effect. As shown in Figure 1A, cell growth continued for over 16 d after subculturing of the cells. PAL activity in untreated cells remained more or less constant over 11 d, but, in PGA lyase-treated cells, PAL activity (measured 7 h after elicitor application) was strongly stimulated (Fig. 1B). The cells were most responsive to elicitor treatment 4 d after subculturing, and responsiveness decreased as the culture aged. After 11 d of growth, little if any inducible activity was observed. Consequently, all further experiments were performed 4 d after subculture. Cultures subjected to weekly subculture for over 1 year retained essentially the same growth and elicitor inducibility characteristics.

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. 2). In addition to a crude PGA lyase preparation, an elicitor prepared from mycelial walls of the soybean fungal pathogen *P. megasperma* f.sp. *glycinea* (Pmg elicitor), which has been shown to elicit defense reactions in many plants (16, 22), and a similar preparation from the mycelial walls of a strain of the phytopathogenic fungus *F. oxysporum* (Fo elicitor) were tested. PAL enzyme activity was assayed 7 h after elicitor application. In each case, PAL activity increased in a dose-dependent manner before reaching a maximum. The decreased PAL activity at high PGA lyase concentrations may be due to loss in cell viability. In some experiments, partially purified PGA lyase was used in place of the crude preparations. The response was similar to that obtained with crude preparations (data not shown). However, although autoclaving the partially purified preparation abolished its elicitor activity, autoclaved crude extracts retained some ability to elicit an increase in PAL activity (data not shown). This suggests that, in addition to PGA lyase, there may be other nonlabile factor(s) in the crude preparation that can act as elicitor(s). Based on these results, further experiments were carried out using 0.15 unit/mL PGA lyase, 100 μg/mL Pmg elicitor, or 75 μg/mL Fo elicitor.

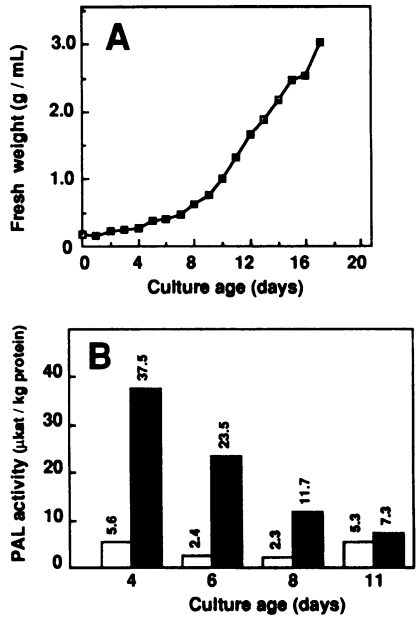

Figure 1. Poplar cell culture growth and elicitor responsiveness. A, Growth curve of the poplar clone H11-11 suspension culture. Culture age refers to days after subculture. B, PAL enzyme activity in untreated control cells (open bars) and cells treated with 0.15 units/mL PGA lyase (filled bars), at various times after subculture. Enzyme activity was measured 7 h after elicitor application.

Kinetics of Phenylpropanoid Biosynthetic Enzyme Induction

Figure 3 shows the kinetics of activation of PAL enzyme activity after exposure to the three elicitors over a 24-h time period. Activation was transiently induced with similar kinetics after treatment with each elicitor. Activities above basal levels were clearly detectable by 3 h and reached maxima 5 to 7 h after application, at which time they were 10- to 20-fold above those in untreated control cells. By 24 h, activities had decreased to only a few fold above basal levels. Some quantitative variation in the responses to the elicitors was observed, with Pmg being the weakest elicitor.

In many systems, increased activity of 4CL, the last enzyme of general phenylpropanoid metabolism, is tightly coordinated with that of PAL after elicitor treatment (5, 10, 16, 24). Figure 4A shows that, in elicitor-treated poplar cells, 4CL enzyme activity was transiently activated by PGA lyase to

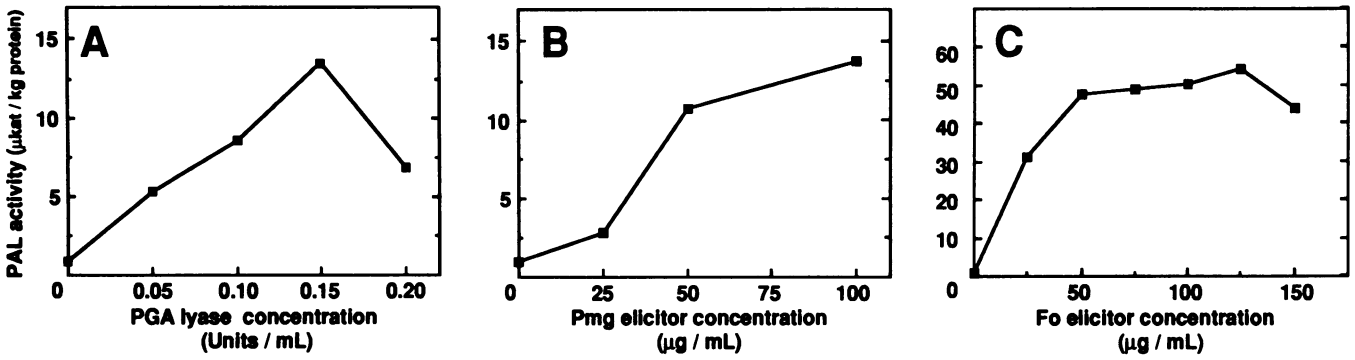

Figure 2. Dose response curves of suspension-cultured cells to three elicitor preparations. A, PGA lyase; B, Pmg; C, Fo. PAL enzyme activity was measured 7 h after elicitor application to 4-d-old cultures. Concentrations of Pmg and Fo elicitors are given in μg of elicitor per mL.

levels about 10-fold above those in untreated cells. The increase in 4CL activity occurred with kinetics very similar to those of PAL, with maximum activity observed at about 7 h after elicitation. Similar results were obtained in Fo elicitor-treated cells (data not shown).

In contrast to PAL, 4CL enzymes are active against several substrates (in addition to 4-coumarate). These are generated by the hydroxylation and methoxylation of 4-coumarate (23). In some plants (*e.g.* parsley and potato; ref. 16), single forms of the 4CL enzyme utilize all such compounds as substrates. In poplar, however, three 4CL isoenzymes have been described that differ markedly in their abilities to utilize 4-coumarate, caffeate, ferulate, and sinapate as substrates (12). We thus tested the ability of the 4CL enzyme activities in untreated and elicitor-treated cells to utilize these compounds as substrates. Figure 4B shows that 4CL enzyme activity in untreated cells was about equally active against 4-coumarate, caffeate, and sinapate and had lower activity against ferulate. In elicitor-treated cells, a different pattern was observed. The increased 4CL enzyme activity in these cells preferentially utilized 4-coumarate and ferulate as substrates; there was little or no increase in 4CL activity capable of utilizing caffeate and sinapate as substrates. These results suggest that elicitor treatment may lead to the activation of a subset of 4CL isoenzymes that preferentially utilize 4-coumarate and ferulate, and that the activities of potential 4CL isoenzymes that preferentially utilize caffeate and sinapate are unaffected by elicitor treatment.

The above results indicated that general phenylpropanoid metabolism is rapidly activated in poplar cells treated with elicitors. Since the biochemical nature of the poplar phenylpropanoid metabolites that are synthesized in response to the elicitors is unknown, it was of interest to determine which, if any, phenylpropanoid branch pathway-specific enzyme activities were induced. Cinnamyl alcohol dehydrogenase enzyme activity (specific to the synthesis of monolignol lignin precursors) was not induced above levels found in untreated cells after treatment with Fo (Fig. 5) or PGA lyase (data not shown). Coniferin β-glucosidase, whose activity is thought to be required to release free monolignols (*e.g.* coniferyl alcohol) from their glycosides (*e.g.* coniferin) in the cell wall before lignin polymerization (23) was also unaffected by elicitor treatment (Fig. 5). In contrast, β-glucosidase activity capable of hydrolyzing glucose from the artificial substrate 4-nitrophenyl-β-glucoside increased dramatically in response to elicitor treatment (Fig. 5). The natural substrate(s) of this activity in poplar cells is unknown at present.

Finally, Figure 5 shows that no increase in CHS enzyme

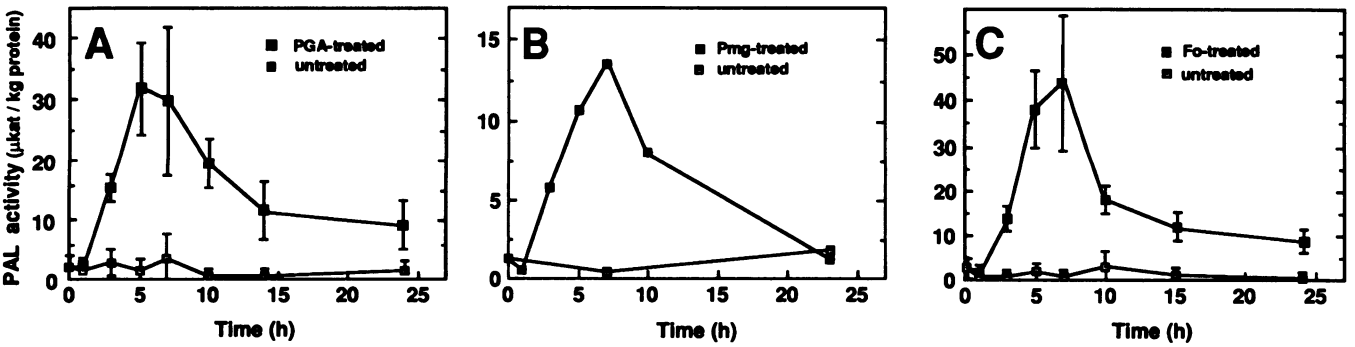

Figure 3. Changes in PAL enzyme activity in elicitor-treated (■) and untreated control (⊡) cell cultures. A, PGA lyase-treated; B, Pmg-treated; C, Fo-treated. Values in A and C are the mean ± SD of four separate experiments; in B, the values represent the average of two separate experiments.

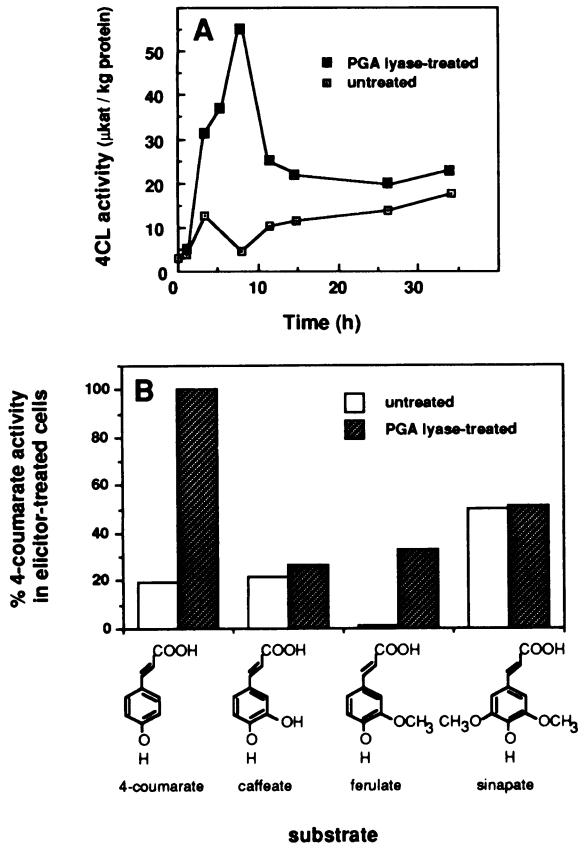

Figure 4. 4CL enzyme activity in elicitor-treated and untreated control cell cultures. A, Changes in 4CL enzyme activity in PGA lyase-treated (■) and untreated control (⊡) cell cultures, using 4-coumarate as a substrate. B, Substrate specificity of 4CL enzyme(s) in elicitor-treated (filled bars) and control untreated cells (open bars). The substrates tested and their structures are given below; activities are given as a percentage of the enzyme activity in elicitor-treated cells measured using 4-coumarate as a substrate.

activity (specific to flavonoid biosynthesis) occurred within 24 h of exposure to PGA lyase or other elicitors (data not shown). These results suggest that the activation of the biosynthesis of flavonoid and lignin precursors is not a major component of the elicitor-triggered defense mechanisms in these poplar cells, but that deglycosylation of glucose conjugates may be part of such defense mechanisms.

Kinetics of PAL and 4CL mRNA Accumulation

We wished to determine whether activation of PAL gene expression precedes the large increase in extractable enzyme activity observed after elicitor treatment. To do so, we first screened a poplar H11-11 cDNA library for PAL-specific clones, using a potato PAL cDNA (10), which cross-hybridized with poplar genomic DNA on Southern blots (data not shown). A total of over 20 positive clones were identified. Partial DNA sequences of several of these showed that they had continuous stretches of similarity to the parsley PAL-1 sequence. The longest clone, H11 PAL.7, was 2.4 kb in size, near that expected for a full length PAL cDNA. The complete nucleotide sequence of this clone will be presented elsewhere (R Subramaniam, C Douglas, manuscript in preparation). Figure 6 shows a homology matrix comparison of a portion of the H11 PAL.7 sequence (between approximately 1400 and 1600 bp with respect to the 5′ end of the clone) with that of a 2462-bp parsley PAL-1 cDNA clone (24). The continuous sequence similarity of this portion of the putative poplar PAL cDNA clone to the corresponding region of the parsley PAL-1 gene and similar results from other sequenced portions of H11 PAL.7 (data not shown) strongly suggest that this clone encodes PAL.

H11 PAL.7 was used as a hybridization probe for Northern blots of total RNA isolated at various times after PGA lyase elicitor treatment of the poplar cell culture. Figure 7A shows that a large and transient increase in the amount of an approximately 2.5-kb RNA hybridizing to the probe was detected by 0.5 h after elicitor treatment. This size is consistent with the size of PAL transcripts in other plants (5, 10, 24), and identically sized RNA was detected on similar Northern blots with the potato PAL cDNA as a probe at reduced stringency (data not shown). Maximum PAL RNA levels were observed 1 to 3 h after the onset of treatment and declined to levels only slightly above those in untreated cells by 10 h. No accumulation of PAL RNA was observed in untreated control cells. The poplar PAL probe also hybridized to a 2.5-kb RNA from young leaves of the poplar clone H11-11 (H11 leaf, Fig. 7A), in which high PAL enzyme activities were observed (R Subramaniam, F Williams, C Douglas, unpublished observations), and which were used as a source of RNA for construction of the cDNA library.

In many systems, activation of 4CL gene expression occurs coordinately with that of PAL genes (5, 10, 16, 24). To determine whether the accumulation of poplar 4CL RNA was coordinately induced with that of PAL RNA, we used a heterologous 4CL probe to detect poplar 4CL transcripts on Northern blots. The probe, a 280-bp fragment of the parsley 4CL-1 cDNA (7), lies within a region conserved between potato and parsley 4CL genes (M Becker-André, personal communication). To increase our ability to detect 4CL transcripts, poly(A^+) RNA was isolated from PGA lyase-treated cells or control cells at selected time points. The probe hybridized to an approximately 2.0-kb RNA in elicitor-treated cells, which accumulated to high levels by 1 to 3 h after elicitor treatment (Fig. 7B). To ensure that equal amounts of mRNA were present in each lane, the blot was subsequently stripped and rehybridized to the cDNA of a parsley gene whose expression is not affected by elicitor treatment (CON2; ref. 32). This probe hybridized with approximately equal efficiency to each lane (data not shown). Furthermore, essentially the same results were obtained with poly(A^+) RNA isolated from cells treated with PGA lyase in a second, independent experiment (data not shown). The RNA detected by the heterologous 4CL probe was of the same size as 4CL transcripts in elicitor-treated parsley cells (data not shown) and also hybridized to a 2.0-kb RNA from H11-11 young leaf tissue (H11 leaf). We are currently using this probe to isolate poplar 4CL cDNA clones. These results indicate that accumulation of both PAL and 4CL mRNAs is rapidly in-

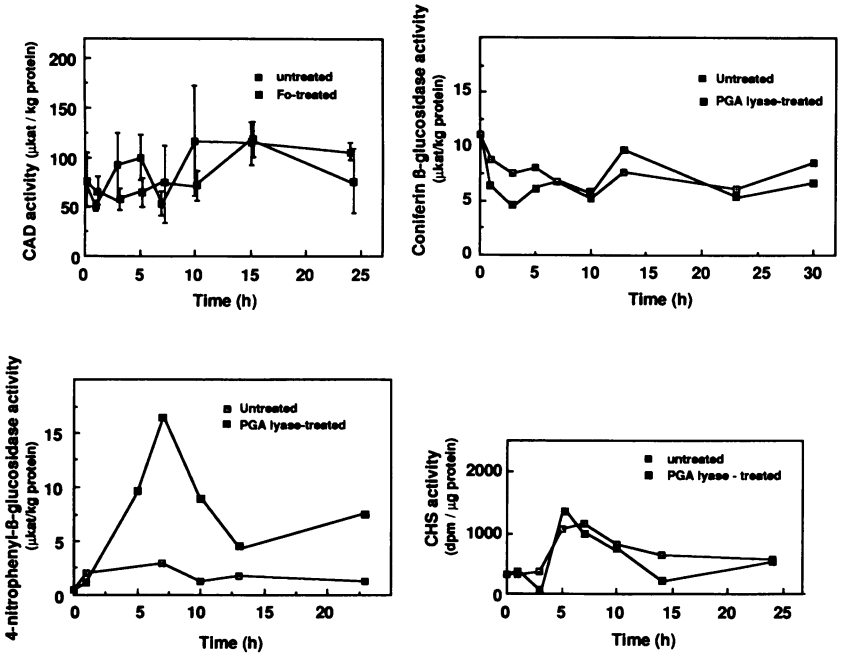

Figure 5. Changes in CAD, coniferin β-glucosidase, 4-nitrophenyl-β-glucosidase, and CHS enzyme activities in elicitor-treated (■) and untreated control (▣) cell cultures. Values for CAD are the mean ± SD of four separate experiments; values for other enzymes are from single experiments, but similar results were obtained in at least three experiments.

duced in elicitor-treated cells, and that mRNA accumulation precedes maximum PAL and 4CL enzyme activities.

Accumulation of Wall-Associated Phenolic Material

By 24 h after elicitor treatment, the poplar cells were noticeably brown, indicative of the deposition of phenolic compounds in the cell walls. We used the thioglycolic acid method (35) to extract and quantify polymeric phenolic material from the cell walls of control and elicitor-treated cells (Fig. 8). A clear difference in the amount of this material was evident as early as 7 h after the onset of the treatment, and by 30 h it had accumulated to levels about fourfold above those in control cells, which stayed constant over the course of the experiment.

While thioglycolic acid extracts lignin and other phenolic polymers form cell walls, lignin-thioglycolic acid complexes can also contain protein (35), and, by itself, the method does not demonstrate the presence of phenolic material in cell walls. To provide further evidence for the accumulation of phenolic material in cell walls of elicitor-treated poplar cells, we stained Fo elicitor-treated cells or untreated control cells with phloroglucinol-HCl. A red coloration with this reagent is indicative of coniferaldehyde end-groups in guaiacyl lignin (23), but the reagent will also react with other phenylpropanoid and phenolic materials in cell walls (25). After 24-h treatment with elicitor, phloroglucinol-stained cells developed an intense red color (Fig. 9). In contrast, no staining was observed in untreated cells harvested at the same time. Washing cells with 50% and 100% methanol before staining had no apparent effect on the intensity of staining (data not shown), indicating that the accumulating phenolic compounds are tightly associated with the walls. However, mild base hydrolysis of the cell walls completely abolished the phloroglucinol staining reaction (data not shown). This sug-

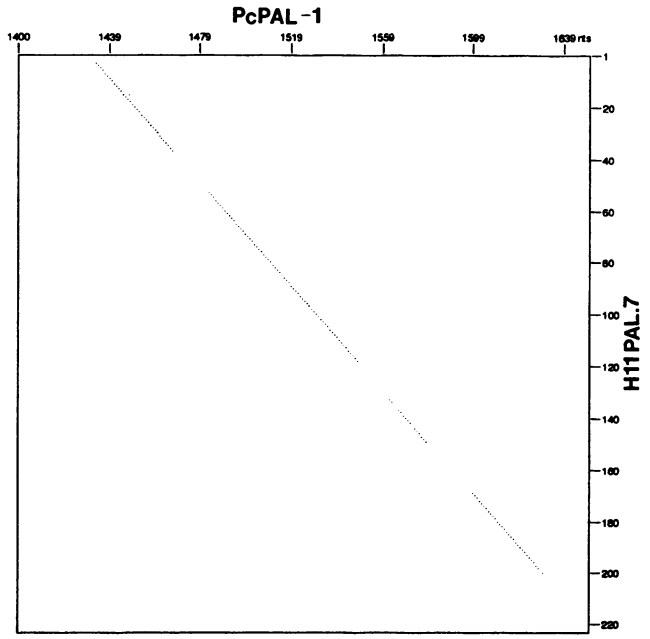

Figure 6. DNA sequence comparison between the parsley PAL-1 gene (PcPAL-1) and a portion of the 2.4-kb hybrid H11 PAL cDNA (H11 PAL.7). The entire PcPAL-1 sequence (2462 bp) was compared to 220 bp of H11 PAL.7 sequence starting at a restriction site approximately 1.4 kb from the 5′ end of the cDNA; only the region showing similarity is shown. The homology matrix computer program used was set with a window of 21 bp and a maximum of 6-bp mismatch allowed.

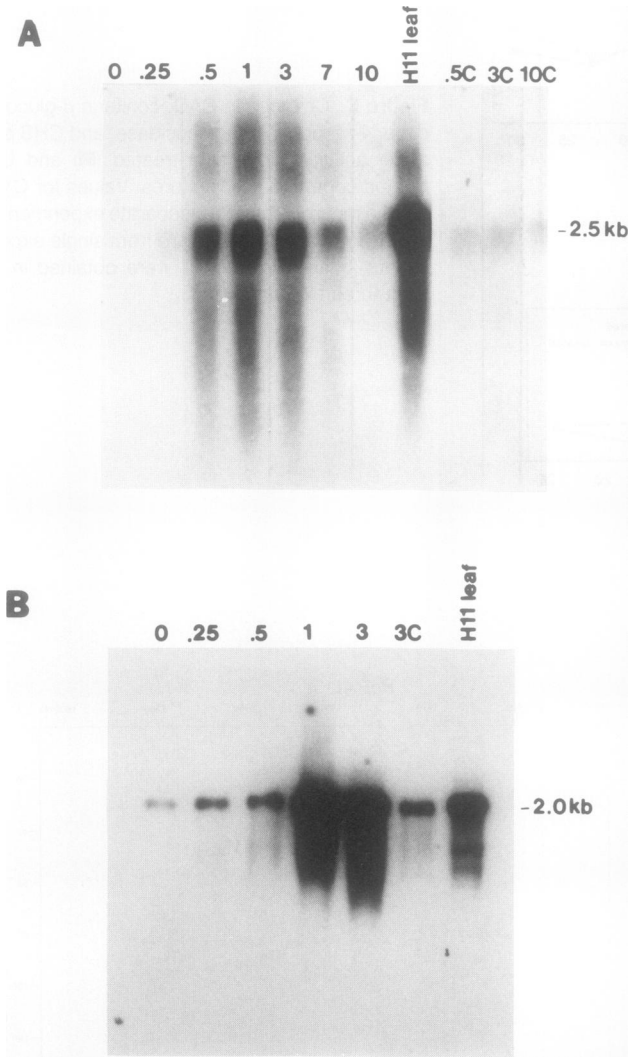

Figure 7. Time courses of PAL and 4CL mRNA accumulation in elicitor-treated cell cultures. A, Homologous H11–11 PAL probe hybridized to an RNA blot of total RNA (10 μg/lane) isolated from cells treated with 0.15 units/mL of PGA lyase for 0 to 10 h, or from untreated cells at 0.5, 3, and 10 h (0.5C, 3C, and 10C). H11 leaf, 3 μg poly(A)$^+$ RNA from young leaves of H11–11 poplar plants. B, Heterologous parsley 4CL probe hybridized to an RNA blot of poly(A)$^+$ RNA (3 μg/lane) isolated from cells treated with 0.15 unit/ml of PGA lyase for 0 to 3 h and from untreated cells at and 3 h (3C). H11 leaf, 3 μg poly(A)$^+$ RNA from young leaves of H11–11 poplar plants. RNA sizes in kb were calculated from migration of RNA standards and from co-migration with cross-hybridizing RNAs from parsley cells (data not shown). Ethidium bromide stained gels were examined before and after transfer to ensure even loading and transfer of RNA in each lane.

gests that the bulk of the phenolic material that accumulates in the cell walls of elicitor-treated cells is not lignin (which would not be released by base hydrolysis) but phenolic material esterified to the cell walls.

DISCUSSION

We show in this article that one response of suspension-cultured cells from the fast-growing *P. trichocarpa* × *P. deltoides* hybrid H11–11 to treatment with elicitors is the rapid activation of phenylpropanoid metabolism. The activation proceeds sequentially from PAL and 4CL mRNA accumulation to large increases in extractable PAL and 4CL enzyme activities and the accumulation of cell wall-bound phenolic metabolites. This response is similar to those observed in cell cultures of a number of herbaceous plants treated with elicitors (2, 5, 16, 22) or in pathogen-infected tissue of such plants (10, 16, 19, 22).

Several aspects of the interaction between elicitors and poplar cells are consistent with observations in other systems: The dependence of poplar cell elicitor responsiveness on the growth stage of the cell culture is similar to that described for parsley cell cultures (20), and the qualitatively similar response of poplar cells to PGA lyase, Pmg, and Fo elicitors is consistent with the responses of suspension-cultured cells of herbaceous plants such as parsley (4, 21), alfalfa (2), and *Arabidopsis* (5) to elicitors of diverse origin. Elicitor activity of *E. carotovora* PGA lyase preparations has been ascribed to the enzymatic release of endogenous plant cell wall fragments that in turn act as elicitors (3). Thus, in poplar, similar to soybean (3), parsley (4), *Arabidopsis* (5), and alfalfa (2), defense reactions appear to be triggered by elicitors of both fungal and endogenous plant cell wall origin.

Extractable PAL and 4CL enzyme activities in elicitor-

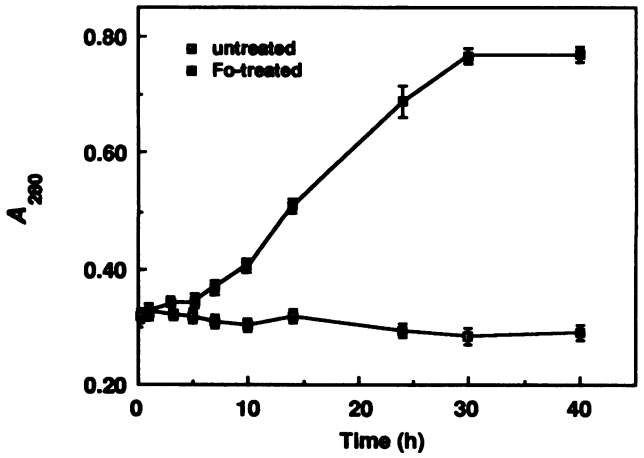

Figure 8. Thioglycolic acid-extractable material in the cell walls of Fo elicitor-treated cells (■) and untreated control cells (□). Values are the mean ± SD of three replicates per time point. The A_{280} of redissolved thioglycolic acid extracts was used to determine relative amounts of the material.

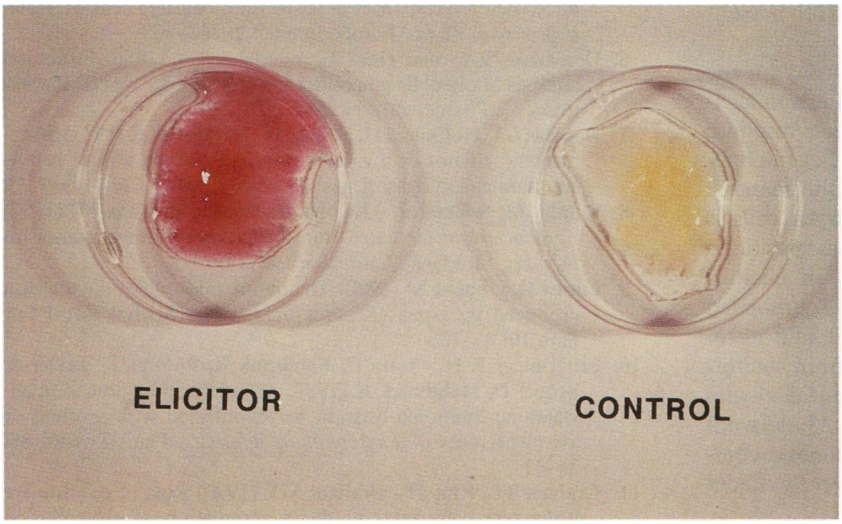

Figure 9. Phloroglucinol-HCl staining of unextracted Fo elicitor-treated cells (ELICITOR) and unextracted untreated control cells (CONTROL) 24 h after the start of elicitor treatment.

treated poplar cells were coordinately and transiently elevated to levels 10- to 20-fold above those in untreated cells (Figs. 3 and 4), and this was preceded by the accumulation of putative poplar PAL and 4CL mRNAs (Fig. 7). This coordinate activation of general phenylpropanoid metabolism has been observed in elicitor-treated cells and/or fungal infected leaf tissue of several plants (5, 10, 19, 24). The apparent rapid activation of poplar PAL and 4CL gene expression is similar in timing to the rapid elicitor-induced activation of PAL mRNA accumulation in elicitor-treated French bean cells and is consistent with the almost immediate elicitor-induced transcriptional activation of bean PAL and CHS genes (22).

In elicitor-treated H11–11 cells, 4CL enzyme activity preferentially utilized 4-coumarate and ferulate as substrates, and no increases in 4CL activity capable of utilizing caffeate or sinapate were observed. A possible explanation for this finding is that 4CL isoform(s) with substrate specificities for 4-coumarate and ferulate accumulate specifically in response to elicitation. Three distinct 4CL isoenzymes have been described in poplar, where they were reported to be distributed in a tissue-specific manner (12). One partially purified poplar 4CL isoenzyme, form II, was reported to exhibit substrate specificity similar to that which we observed in elicitor-treated cells: 4-coumarate and ferulate were utilized, but conversion of caffeate and sinapate into their CoA esters was not detected (12). Biochemical characterization of potential 4CL isoforms in control and elicitor-treated H11 cell cultures will be required to definitively establish whether there is differential activation of 4CL isoforms, but to our knowledge this is the first report indicating that elicitor treatment may lead to the differential activation of 4CL isoforms with distinct catalytic properties. Evidence suggesting the differential induction of 4CL isoenzymes with different substrate specificities has been obtained in maize plants infected with *Helminthosporium maydis* (34). It was suggested that such isoenzymes could regulate the synthesis of different phenylpropanoid products in susceptible and resistant interactions of maize with *H. maydis* (34). In poplar, such isoforms could be potentially important in metabolic channeling of phenylpropanoid metabolism by controlling the types of cinnamoyl-CoA esters formed.

The nature of potential defensive compounds that accumulate in the elicitor-treated poplar cell cultures is currently under investigation in our laboratory. Elicitor activation of general phenylpropanoid metabolism suggests that phenylpropanoid derivatives are likely to accumulate in the treated cell cultures, and preliminary data suggest that one major accumulating soluble metabolite is the glucoside of a small phenolic compound (P Spencer, C Douglas, unpublished observations). The lack of elicitor-induced changes in the activities of two enzymes specific to lignin biosynthesis, or changes in the activity of CHS, specific to flavonoid biosynthesis, suggests that induced lignification and accumulation of flavonoids may not be major elicitor-triggered defense responses in this poplar hybrid.

Interestingly, β-glucosidase activity capable of hydrolyzing glucose from the substrate 4-nitrophenyl-β-glucoside was rapidly and strongly induced in elicitor-treated cells (Fig. 5). While we do not yet know the natural cellular substrate(s) of this activity, it is notable that poplar and other species in the Salicaceae in general contain large numbers of phenolic and phenylpropanoid glycosides (6, 14, 27). Potentially, increased levels of β-glucosidases could hydrolyze the glycosidic linkages of such compounds to release aglycones with defensive roles. Rapid hydrolysis of constitutively produced isoflavonoid conjugates in response to infection has been shown to be a potentially important defense reaction in incompatible interactions between soybean and *P. megasperma* f.sp. *glycinea* (11).

A major site for the accumulation of phenylpropanoid metabolites in elicitor-treated poplar cells appears to be the cell wall, based on a large (but not quantified) increase in phloroglucinol-HCl staining material in the walls of elicitor-treated cells (Fig. 9) and on the accumulation of thioglycolic acid-extractable wall-bound material in elicitor-treated cells relative to levels in control cells (Fig. 8). These observations are consistent with the elicitor-induced deposition of a variety

of phenylpropanoid compounds in the walls of other plant cells (16).

Thioglycolic acid extraction has been used to assay for the accumulation of lignin and "lignin-like" compounds in elicitor-treated castor bean cells (1), which showed a response similar to that which we observed in poplar cells. Several lines of evidence suggest, however, that the bulk of the material accumulating in the walls of elicitor-treated poplar cells is unlikely to be lignin: the activities of two enzymes specific to lignin biosynthesis did not increase markedly in elicitor-treated cells; mild base hydrolysis of the cell walls eliminated their ability to be stained by phloroglucinol-HCl, and nitrobenzene oxidation of base hydrolyzed cell walls from elicitor-treated cells yielded very low amounts of the benzaldehydes that would correspond to lignin monomers (ref. 23; data not shown). The nature of the cell wall-bound phenolic compounds that do appear to accumulate is currently under investigation.

Cell cultures have proven useful for investigating certain features of the interactions between herbaceous plants and their pathogens (16). In several cases, the elicitor-induced accumulation of defensive secondary metabolites in cell cultures correlates well with the roles of such metabolites as phytoalexins in whole plant-pathogen interactions (2, 16, 19, 22). We plan to exploit this woody plant cell culture system to identify secondary metabolites whose accumulation is elicitor triggered, and to test the roles such compounds may play in poplar plants in resistance against serious poplar fungal pathogens such as *Melampsora medusae, Melampsora occidentalis, Septoria musiva,* and *Venturia populina.* The system should also prove useful in further studies on the regulation of phenylpropanoid metabolism in poplar.

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