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Stress Responses in Alfalfa[1]

XXI. Activation of Caffeic Acid 3-*O*-Methyltransferase and Caffeoyl Coenzyme A 3-*O*-Methyltransferase Genes Does Not Contribute to Changes in Metabolite Accumulation in Elicitor-Treated Cell-Suspension Cultures

Weiting Ni[2], Vincent J. H. Sewalt, Kenneth L. Korth, Jack W. Blount, G. Murray Ballance[3], and Richard A. Dixon*

Plant Biology Division, Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, Oklahoma 73402

Transcription of genes encoding L-phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway, and caffeic acid 3-*O*-methyltransferase (COMT) and caffeoyl CoA 3-*O*-methyltransferase (CCOMT), enzymes involved in the synthesis of lignin and wall-esterified phenolic compounds, was strongly activated in elicitor-treated cell-suspension cultures of alfalfa (*Medicago sativa* L.). However, consequent changes in the extractable activities of COMT and CCOMT were small to nonexistent compared with a 15- to 16-fold increase in PAL activity. Only low levels of COMT and CCOMT transcripts were reflected in the total and polysomal RNA fractions compared with PAL transcripts. Elicited cell cultures did not accumulate lignin or the products of COMT and CCOMT in the soluble and wall-esterified phenolic fractions. In one alfalfa cell line in which elicitation resulted in very high PAL activity and increased deposition of methoxyl groups in the insoluble wall fraction, there was still no change in COMT and CCOMT activities. Overall, these results indicate that the initial gene transcription events in elicited cells may be less selective than the subsequent metabolic changes, highlighting the importance of posttranscriptional events in the control of phenylpropanoid biosynthesis.

Exposure of plant cells to elicitor macromolecules results in a massive switch in metabolism, leading to the accumulation of low-molecular-weight compounds and proteins that have been ascribed roles in plant defense against pathogens (Collinge and Slusarenko, 1987; Dixon and Harrison, 1990). In some species, such as cucumber and wheat, elicitation results in the increased accumulation of lignin and other cell-wall phenolic materials (Pearce and Ride, 1982; Robertsen, 1986) that may function as physical barriers against pathogen ingress (Vance et al., 1980). In legumes such as bean, soybean, chickpea, and alfalfa (*Medicago sativa*) a major response to elicitation is the accumulation of antimicrobial isoflavonoid phytoalexins (Dixon and Harrison 1990; Dixon et al., 1995).

In alfalfa cell-suspension cultures elicitor-induced accumulation of the isoflavonoid phytoalexin medicarpin is preceded by increases in the extractable activities of all of the enzymes involved in its biosynthesis from L-Phe (Dixon et al., 1995). In addition, elicitation induces other pathways of secondary metabolism, such as the formation of a methoxy-chalcone nodulation gene inducer (Maxwell et al., 1993) and primary metabolic reactions linked to isoflavonoid synthesis. The latter include the pentose phosphate pathway (for supply of NADPH) (Fahrendorf et al., 1995), *S*-adenosyl-L-Met synthase (for supply of methyl group donors) (Gowri et al., 1991a), and acetyl CoA carboxylase (to provide malonyl CoA, a co-substrate of the chalcone synthase reaction) (Shorrosh et al., 1994). In the above cases, de novo transcriptional activation leads to large increases in steady-state transcript levels, followed by large increases in enzymatic activity (from 3- to 30-fold) (Dixon et al., 1995). The kinetics of transcriptional activation of different genes in response to elicitor application suggests that more than one signal pathway is involved in orchestrating the overall transcriptional changes (Ni et al., 1996).

In contrast to the enzymes of phytoalexin biosynthesis, activities of two enzymes of lignin and wall-bound phenolic synthesis, COMT and cinnamyl alcohol dehydrogenase, were only weakly induced in elicitor-treated alfalfa cultures. This response appeared to correlate with the accumulation of small amounts of wall-bound phenolic material, the identity of which was not determined (Dalkin et al., 1990; Gowri et al., 1991a). Since the methods previously used to measure wall-bound phenolic compounds in elicited cell cultures may lead to overestimates (Lewis and Yamamoto, 1990), we have now reevaluated whether elicitation results in changes in lignin and wall-esterified phenolics by using more rigorous methods of analysis. We now demonstrate that the genes encoding two lignin *O*-

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[2] Present address: U.S. Department of Agriculture, Agricultural Research Service, Department of Agronomy and Genetics, University of Minnesota, St Paul, MN 55108.

[3] Permanent address: Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.

* Corresponding author; e-mail radixon@noble.org; fax 1–405–221–7380.

Abbreviations: CCOMT, caffeoyl CoA 3-*O*-methyltransferase; COMT, caffeic acid 3-*O*-methyltransferase; IFR, isoflavone reductase; NDF, neutral detergent fiber; PAL, L-Phe ammonia-lyase.

methyltransferases, COMT and CCOMT, are transcriptionally activated to high levels in elicited alfalfa cell cultures, but that this leads to little if any increase in extractable enzymatic activity or products of lignin monomer synthesis in the lignin or in the soluble and wall-bound phenolic fractions. In contrast, transcriptional activation of genes encoding PAL, the first enzyme of phenylpropanoid synthesis, results in large increases in enzymatic activity, which, in combination with strong induction of IFR, an enzyme specific for isoflavonoid phytoalexin biosynthesis in elicited cells, leads to corresponding accumulation of the phytoalexin medicarpin. Thus, more genes are activated in the initial transcriptional response to elicitor than are involved in the subsequent metabolic response, suggesting that in alfalfa suspension cells the overall pattern of gene expression is functionally determined at the posttranscriptional level.

MATERIALS AND METHODS

Growth and Elicitation of Cell Cultures

Several different batches of alfalfa (*Medicago sativa* L. cv Apollo) cell cultures were used in these experiments. The cultures were initiated from stock callus lines that had been maintained by regular subculture over a period of at least 5 years. Line A12 was initiated from callus stock approximately 12 months after line A1. Experiments were performed on newly initiated suspension lines from the fourth passage on. Suspension cultures were maintained and exposed to yeast elicitor as previously described (Dalkin et al., 1990). Cells were harvested at various times by vacuum filtration on nylon mesh, frozen in liquid N_2, and stored at −70°C.

cDNA Probes

The PAL probe used for northern blot analysis and detection of in vitro transcripts was the 0.47-kb internal *Hin*dIII fragment of the alfalfa pAPAL1 cDNA clone (Gowri et al., 1991b). The alfalfa COMT probe was the 1.3-kb insert from plasmid pCOMT1 (Gowri et al., 1991a). The CCOMT probe was a full-length alfalfa CCOMT sequence (G.M. Ballance and R.A. Dixon, unpublished data; GenBank accession no. U20736). The IFR probe was the full-length insert from plasmid pIFRalf1 (Paiva et al., 1991).

Isolation and Analysis of RNA

Total RNA was isolated from frozen cells according to Chomczynski and Sacchi (1987). Polysomal RNA was isolated using the procedure of Mignery et al. (1984). RNA blot analysis (10 μg of RNA per lane) was performed by standard procedures (Sambrook et al., 1989). Probes used for total and polysomal RNA blot analysis were produced by random-primer labeling, and were of approximately equal length and specific activity. An rRNA probe was used as a control for loading and transfer efficiency. Relative densities of bands on autoradiographs were obtained by scanning the films with an image-analysis system (Java, Jandel Scientific, Corte Madera, CA) or by phosphorimager analysis.

Isolation of Nuclei and Run-On Transcription

Nuclei were isolated at 4°C by a modification of the procedure of Kodrzycki et al. (1989). Frozen cells (10 g per time point) were ground to a fine powder in a mortar in liquid N_2. The powder was then suspended in 50 mL of Honda buffer (440 mM Suc, 25 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 2.5% [w/v] Ficoll, 5% [w/v] dextran, and 10 mM β-mercaptoethanol) and filtered through two layers of Miracloth (Calbiochem), and large starch grains were removed by centrifugation at 100*g* for 5 min. Nuclei were pelleted by centrifugation at 1200*g* for 5 min, resuspended in Honda buffer, centrifuged as above, and resuspended in nuclei washing buffer (50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 20% [v/v] glycerol). The nuclei were pelleted and resuspended twice more, with the final resuspension in nuclei washing buffer containing 50% (v/v) glycerol. They were then divided into small aliquots, frozen in liquid N_2, and stored at −80°C. Their purity and integrity were monitored microscopically.

Run-on transcription was performed essentially as described by DeLisle and Crouch (1989) in a total volume of 200 μL. Reaction mixtures contained 80 μL of nuclei, 100 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 500 μM ATP, GTP, and CTP, 30 μM UTP, 200 μCi of [α-^{32}P]UTP, 0.1 mM phosphocreatine, 25 ng/mL phosphocreatine kinase, and 500 units of ribonucleose inhibitor (RNasin, Promega). Reactions were incubated at 30°C for 30 min and stopped with 5 volumes of 2% (w/v) SDS and 20 mM EDTA. RNA transcripts were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v), precipitated with ethanol, and resuspended in water. Unincorporated radioactive UTP was removed with a spin column (Sephadex G-50, Sigma). Transcription was linear over the 30-min incubation period, and pulse-chase experiments revealed no degradation of total transcripts over this period.

Samples (0.5 μg) of PAL, COMT, and CCOMT cDNA inserts were denatured with 0.2 N NaOH and slot-blotted onto a nylon membrane (GeneScreen-Plus, NEN Research Products) for hybridization to ^{32}P-labeled in vitro transcripts. Hybridization was at 65°C overnight in 1% SDS, 10% dextran sulfate, and 1 M NaCl, followed by two washes for 15 min at room temperature in 2× SSC, two washes for 30 min in 2× SSC/1% SDS, and one final wash for 15 min at 55°C in 0.1× SSC. Autoradiographs were quantitated using a phosphorimager.

Enzyme Extraction and Assays

PAL activity was measured as described previously (Dalkin et al., 1990). For assay of *O*-methyltransferase activities, frozen suspension cells were ground to a fine powder under liquid N_2 using a mortar and pestle. Powdered cells were extracted for 20 min at 4°C in 10 mM Tris-HCl, pH 7.35, 0.2 mM $MgCl_2$, 2.0 mM DTT, and 10% (v/v) glycerol. After centrifugation at 12,000*g* at 4°C for 10 min the extract was desalted on a PD-10 column (Pharmacia).

For subsequent assays, aliquots of desalted extract were adjusted to pH 7.2 (COMT) or pH 7.5 (CCOMT) by adding one-tenth volume of 1 M Tris-HCl of the appropriate pH. Soluble protein in the enzyme extracts was determined using the Bradford dye-binding reagent (Bio-Rad) with BSA as the standard.

COMT activity was assayed as described previously (Gowri et al., 1991a). CCOMT was assayed by incubation of extracts with [$^{14}CH_3$]*S*-adenosyl-L-Met and caffeoyl CoA. Caffeoyl CoA was prepared from caffeic acid according to the method of Stöckigt and Zenk (1975). The assay mixture contained 5 μL of [$^{14}CH_3$]*S*-adenosyl-L-Met (0.6 mM, 13 μCi/μmol), 5 μL of caffeoyl CoA (1.05 mM), 15 μL of assay buffer (100 mM Tris-HCl, pH 7.5, 0.2 mM $MgCl_2$, 2.0 mM DTT, 10% glycerol), and 25 μL of desalted, pH-adjusted extract, and was incubated for 30 min at 30°C. The reaction was stopped by adding 6 μL of 5 N NaOH, and CoA esters were hydrolyzed by incubation at 40°C for 15 min. The solution was then acidified by adding 44 μL of 1 N HCl, and the labeled ferulic acid was extracted into 250 μL of hexane:ethyl acetate (1:1, v/v). After a brief centrifugation to separate phases 180-μL aliquots of the organic phases were transferred to scintillation vials for determination of radioactivity. Blank values (from reactions stopped at time 0) were subtracted.

Determination of Soluble Phenolic Compounds

Cells (0.25–0.35 g wet weight) were extracted in 8 mL of acetone at room temperature with shaking for 24 h. The acetone was dried under N_2, and the residue was redissolved in methanol for HPLC analysis of medicarpin (Blount et al., 1993). Other soluble phenolics were determined by analysis of HPLC traces at 235 and 310 nm, followed by UV diode-array spectroscopic analysis with comparison against authentic standards.

Determination of Lignin and Wall-Bound Phenolic Acids

For determination of phenolic acids, the solid residues after enzyme extraction or extraction of soluble phenolics were lyophilized, washed twice with acetone, and subsequently subjected to saponification in 4 mL of 1 N NaOH at room temperature in 15-mL tubes. After flushing with N_2 for 1 h, the tubes were shaken at room temperature for another 20 h. The supernatant was removed after centrifugation (10,000*g* at 4°C for 10 min), and the residue was washed twice with 2 mL of 0.2 N NaOH. The combined supernatant and washings were acidified with 2 N HCl to pH 1.0 and extracted three times with 10 mL of ethyl acetate. Phases were separated by centrifugation at 3,000*g* at 4°C for 10 min. The combined ethyl acetate extracts were evaporated under N_2 at room temperature, and derivatized with 200 μL of *N,O*-bis(trimethylsilyl) acetamide at 80°C for 10 min. A 50-μL aliquot was dissolved in 0.9 mL of ethyl acetate and subjected to GC-MS analysis. Authentic standards (cinnamic, *p*-coumaric, ferulic, caffeic, and sinapic acids) were subjected to the same saponification and derivatization procedure and were assayed for reference. Alternatively, ethyl acetate extracts were directly analyzed by HPLC as described above.

For determination of Klason lignin (gravimetrically determined insoluble lignin), cells were lyophilized and extracted with boiling neutral detergent (Robertson and Van Soest, 1980) using filter bags in a batch fiber analyzer (ANKOM, Fairport, NY). The residual NDF was oven-dried at 55°C and used for quantitation of Klason lignin according to the method of Kaar et al. (1991), modified to a microanalytical scale. One-hundred milligrams of NDF was suspended in 1 mL of 72% H_2SO_4 in 50-mL reaction tubes kept in a water bath at 30°C for 1 h. The initial hydrolysis was followed by dilution to 4% H_2SO_4 and autoclaving at 121°C for 1 h. The hydrolysis mixture was passed through a previously tared glass-fiber filter (grade 934 AH, particle retention 1.5 μm, Whatman) in a tared, 30-mL Gooch crucible of fine porosity (pore size 4–5.5 μm). The residue (Klason lignin) and filter were oven-dried at 105°C overnight and weighed. Thioglycolic acid lignin was determined by a modification of the method of Doster and Bostock (1988) as described by Ni et al. (1994). Values were converted from absorbance to percentage of dry matter using the extinction coefficient of 9.4 g^{-1} L cm (Doster and Bostock, 1988). Phloroglucinol staining was carried out as previously described (Ni et al., 1994).

Pyrolysis GC-MS of cell-wall preparations was performed essentially as described by Ralph and Hatfield (1991). Ground, dried cell-wall material (50–150 μg) was pyrolyzed in a quartz tube using a pyrolysis power generator (Pyroprobe 1000, CDS Analytical, Oxford, PA) at 700°C (>20°C/ms ramp) for 10 s using helium as a carrier gas and with a mean linear velocity of 15.4 cm/min. The sample was partitioned with a capillary dimethylpolysiloxane column (DB-1, J&W Scientific, Folsom, CA) (0.25 mm × 60 m × 1.0 μm) in a gas chromatograph (model 5890 II, Hewlett-Packard) in the splitless mode. The initial temperature was set at 50°C for 2 min to trap and focus the volatile components, and was then increased at 40°C/min to a final temperature of 275°C and held for a total run time of 63 min. Compounds were detected with a mass selective detector (model 5971A, Hewlett-Packard) and data were collected and analyzed by the ChemStation DOS program (Hewlett-Packard) for reconstruction of total ion chromatograms.

Lignin methoxyl groups were determined by reaction with 57% hydriodic acid in a methoxyl apparatus heated in an oil bath to 148 ± 1°C, followed by titration with sodium thiosulfate, according to standard T 209 su-72 of the Technical Association of the Pulp and Paper Industry (TAPPI, 1972).

RESULTS AND DISCUSSION

Changes in Lignin *O*-Methyltransferase and PAL Activities in Elicited Cell Cultures

PAL, which catalyzes the deamination of Phe to *trans*-cinnamic acid, is the first committed step in the synthesis of all phenylpropanoid compounds in plants. Lignin monomers and wall-esterified hydroxycinnamic acids are formed by subsequent hydroxylation and methoxylation

of the cinnamic acid nucleus. It has been shown that methylation of the phenolic hydroxyl groups of monolignols can occur at the level of the free acid (catalyzed by a bifunctional caffeic acid/5-hydroxyferulic acid 3-*O*-methyltransferase, COMT) or of the corresponding CoA ester (catalyzed by CCOMT) (Pakusch et al., 1989). COMT converts caffeic acid to ferulic acid and 5-hydroxyferulic acid to sinapic acid, whereas CCOMT converts caffeoyl CoA to feruloyl CoA (Fig. 1). Although the CCOMT reaction was originally believed to be specific for the synthesis of wall-esterifed ferulic acid polymers, it has recently been suggested that it may also be important in the synthesis of lignin (Ye et al., 1994).

We first determined the extractable activities of COMT and CCOMT, and compared them to those of PAL in alfalfa cell-suspension cultures exposed to yeast elicitor, a treatment that results in rapid activation of genes encoding enzymes of the central phenylpropanoid pathway and the flavonoid/isoflavonoid branch pathway (Dixon et al., 1995). Basal enzyme activity levels and the extent of elicitation can vary considerably between different cell culture lines, and we therefore repeated these and subsequent experiments in different cell lines. The data in Figure 2 show results from two cell lines: line A1 shown in Figure 2, A to C, has significantly lower enzymatic activities than line A12 shown in Figure 2, D to F. In both cases, PAL activity began to increase by 1 h postelicitation, attaining a 15- to 16-fold increase by 8 to 9 h. In line A1, COMT activity increased less than 50% by 9 h postelicitation (Fig. 2B), and the overall change in CCOMT activity was only approximately 2-fold, from a lower basal activity than that of COMT (Fig. 2C). This represents a significantly weaker and slower induction of CCOMT enzymatic activity than that observed in elicitor-treated parsley cell cultures (Kühnl et al., 1989), which accumulated wall-bound ferulate esters. In line A12 (Fig. 2, D–F), the basal activities of the three enzymes were around 10-fold higher than those in line A1, but elicitation led to no increase in either COMT or CCOMT activities. These results are in agreement with previous studies that indicate only weak elicitation of COMT activity in alfalfa cell cultures (Jorrin and Dixon, 1990; Gowri et al., 1991a, 1991b). We observed a similar level of PAL induction to that reported here with no accompanying increase in COMT activity in other alfalfa cell lines (data not shown). In pine cell cultures elicitation resulted in a 10-fold induction of PAL activity and a 3-fold induction of COMT activity by 18 to 24 h postelicitation, leading to significant increases in wall-associated, lignin-like material (Campbell and Ellis 1992b).

Lignin *O*-Methyltransferase Genes Are Transcriptionally Activated in Elicited Alfalfa Cells

Nuclear run-on transcription assays revealed that elicitation induced strong increases in the rates of transcription of PAL, COMT, and CCOMT in alfalfa cell-suspension cultures (Fig. 3, A and B). Comparison of the induction kinetics of COMT with those of PAL indicated a distinct lag period of 30 to 45 min before COMT transcription increased to above the level observed at time 0 (Fig. 3A). In contrast, PAL transcription began to increase as early as 10 min postelicitation. The relative levels of transcription of PAL and COMT, normalized to PAL at the time of maximum transcription rate, were 100 and 29 to 35%, respectively, based on duplicate experiments using different alfalfa cell batches, including line A1. Transcription of CCOMT was stimulated to an absolute level of just under 150% that of PAL at 2 h postelicitation, but was delayed by approximately 45 min compared with that of PAL (Fig. 3B). These values for transcription rates of COMT and CCOMT relative to PAL are based on absolute counts incorporated and are not corrected for transcript sizes (the PAL transcript is 2.4 kb and the COMT and CCOMT transcripts are 1.6 and 1.0 kb, respectively); therefore, these values are underestimates. In an independent experiment we observed a rate of CCOMT transcription at 80% the rate of PAL transcription at 2 h postelicitation.

We next determined the effects of elicitation on the steady-state levels of PAL, COMT, and CCOMT transcripts in the total RNA fraction from several alfalfa cell lines (Fig. 4). Both PAL and CCOMT transcripts were transiently induced above the low levels present in control cells, with the increase in

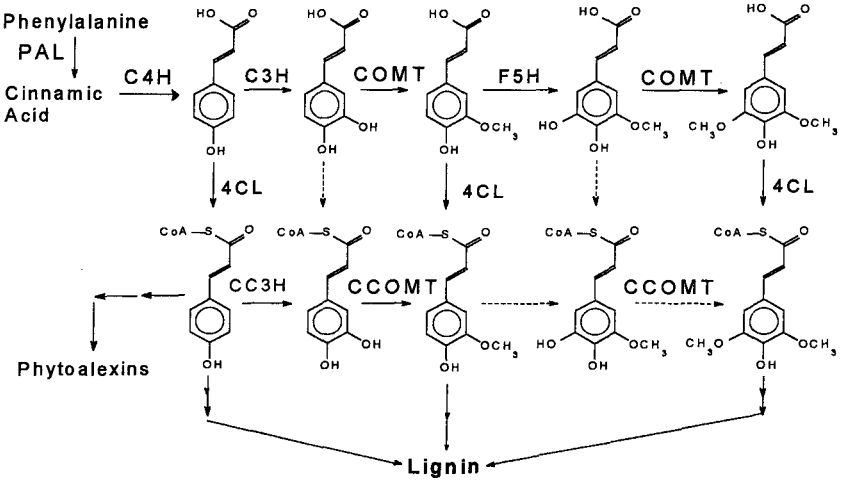

Figure 1. The COMT and CCOMT reactions in relation to the biosynthesis of lignin and isoflavonoid phytoalexins in alfalfa. The other enzymes are: PAL; cinnamate 4-hydroxylase (C4H); coumarate 3-hydroxylase (C3H); ferulate 5-hydroxylase (F5H); 4-coumarate CoA ligase (4CL); and coumaroyl CoA 3-hydroxylase (CC3H).

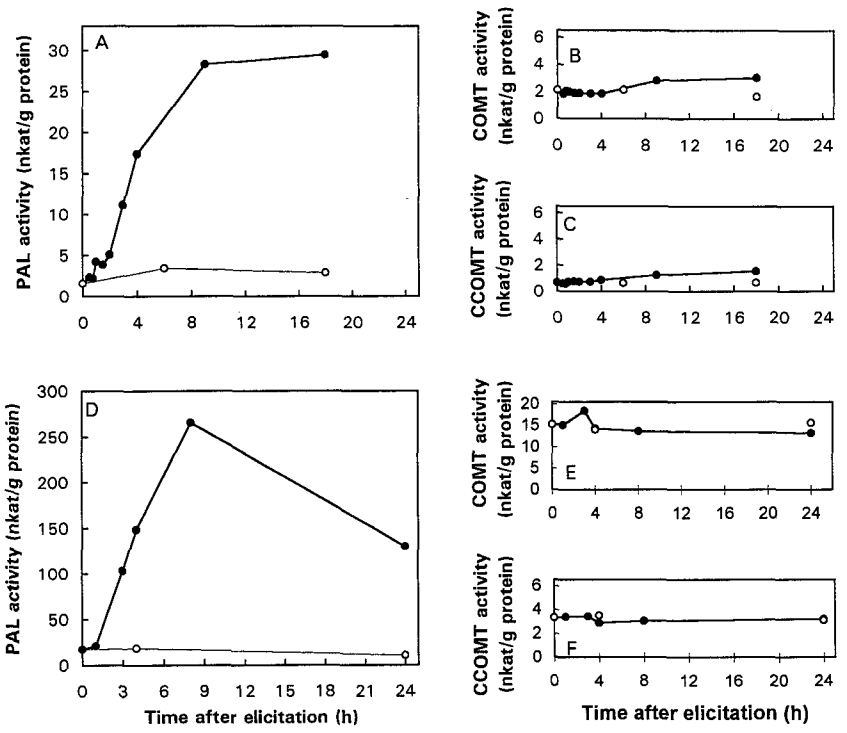

Figure 2. Changes in the extractable activities of PAL, COMT, and CCOMT in elicitor-treated (●) and control (○) alfalfa cell-suspension cultures. The data show results for two different alfalfa cell culture lines, A1 (A–C) and A12 (D–F), which have different basal enzyme activities.

PAL transcripts preceding that of CCOMT transcripts, consistent with the relative transcription kinetics shown in Figure 3B. In contrast, COMT transcripts were present at a relatively high level in unelicited cells, and appeared to decrease in response to elicitor. We also include data in Figure 4B for IFR transcript levels, which, although more strongly induced, parallel those of CCOMT. IFR is a late enzyme in the biosynthesis of the isoflavonoid phytoalexin medicarpin, a major end product of elicitor-induced phenylpropanoid metabolism in alfalfa cells. We have previously demonstrated that IFR transcription is rapidly induced in alfalfa cells, with similar kinetics (relative to PAL) to that of CCOMT transcription shown in Figure 3B (Ni et al., 1996), and that this results in a corresponding increase in IFR enzymatic activity (Paiva et al., 1991).

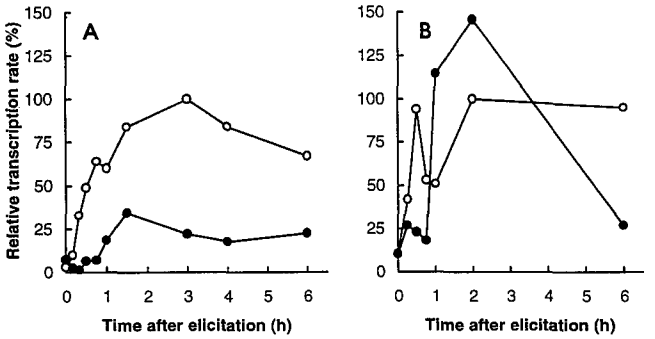

Figure 3. Relative transcription rates of COMT (A) and CCOMT (B) (●) in relation to PAL (○) in elicitor-treated alfalfa cell-suspension cultures (line A1) over the first 24 h postelicitation. Transcription rates are normalized to the maximum values for PAL transcription during the time course (expressed as 100%). The CCOMT analyses were performed on cells from a later passage of the A1 suspension line than those used for the COMT analyses.

Although the induction pattern of CCOMT transcripts shown in Figure 4B appears similar to that of PAL and IFR, quantitation of the blot by phosphor-imaging and correction for the different exposure times revealed that the level of COMT and CCOMT transcripts compared with the level of PAL transcripts at 4 h postelicitation was only 6 and 3%, respectively, assuming equal transfer and hybridization efficiency (Fig. 4C). Furthermore, there was a greater than 40-fold increase in PAL transcripts upon elicitation, whereas the increase in CCOMT transcripts was around 6-fold, and COMT transcripts were lower in elicited cells than in control cells. Thus, in spite of transcription rates comparable to that of PAL, COMT and CCOMT transcripts accumulate to a much lesser extent, suggesting that they might be less stable than PAL transcripts in elicited cells.

Polysomal Recruitment of Elicitor-Induced Transcripts

We analyzed the relative levels of PAL, COMT, CCOMT, and IFR transcripts associated with polysomes to determine whether differential polysomal recruitment might also account for the differences in subsequent activities of the enzymes in spite of their similar transcription rates. Northern blot analysis of polysome-bound mRNAs from two different alfalfa cell lines indicated that PAL transcripts were recruited into polysomes at high levels by 3 h postelicitation, whereas COMT transcripts remained at a similar level in elicited and unelicited cells (Fig. 5), consistent with the levels of total cellular RNAs (Fig. 4). Likewise, the pattern of CCOMT and IFR transcripts in the polysomal fraction reflected their levels in the total RNA fraction.

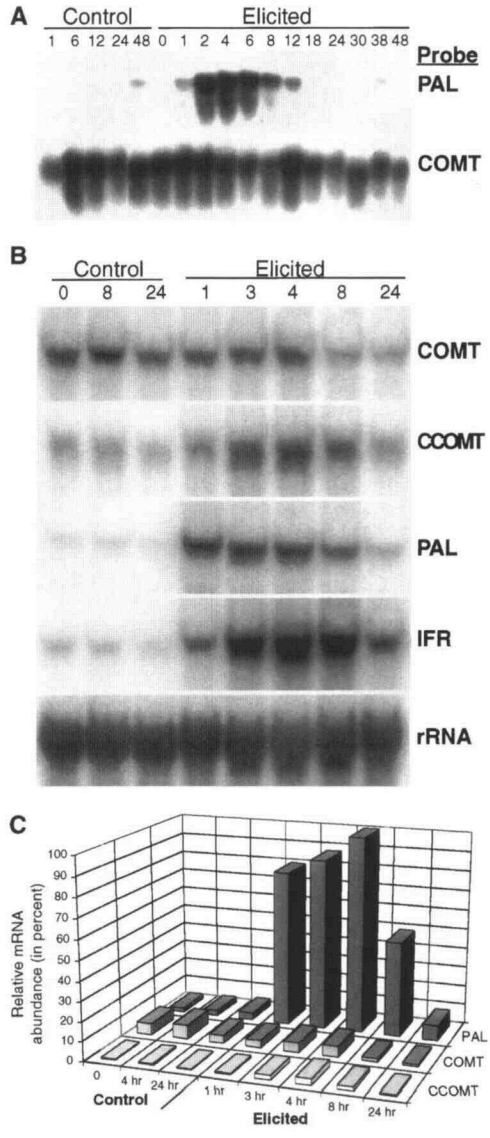

Figure 4. Northern blot analysis of COMT, CCOMT, and PAL transcripts in the total RNA fraction from control and elicitor-treated alfalfa cell cultures at the times (in h) shown. The cells analyzed in A were from line A1, and those analyzed in B were from line A12. Exposure times were: A, PAL 6 h, COMT 48 h (the blot was first probed with COMT and then stripped and reprobed with PAL); B, PAL and IFR 30 min, COMT and CCOMT 16 h. C, Relative levels of PAL, COMT, and CCOMT transcripts obtained by phosphor-imager analysis of the blot in B, with values normalized to the maximum value for PAL as 100%.

It is therefore unlikely that differential polysomal recruitment can explain the different relationships between transcript levels and enzymatic activity for the lignin *O*-methyltransferases compared with PAL and IFR.

Levels of Soluble or Wall-Associated Products of the COMT and CCOMT Reactions in Elicited Cells

A previous study showed that there was no net increase in a small pool of soluble ferulic acid in elicited alfalfa suspension cells measured between 2 and 10 h postelicitation (Orr et al., 1993). Using the cell batches analyzed in Figures 4 and 5, we could detect no ferulate or sinapate derivatives as free acids, esters, or glucosides in the soluble phenolic fraction from control or elicited cells. The major compounds detected were flavonoids, isoflavonoids, and traces of benzoic acid. There was, however, a large increase in the level of medicarpin in elicited cells (Table I), as observed in previous studies (Dixon et al., 1995).

We have reported that elicitation of alfalfa cells led to a 2-fold increase in wall-bound phenolic material as measured by alkaline hydrolysis of the cell-wall hemicellulosic fraction followed by UV spectrophotometry (Dalkin et al., 1990). This wall-bound material was not chemically identified, and, in view of the lack of significant induction of

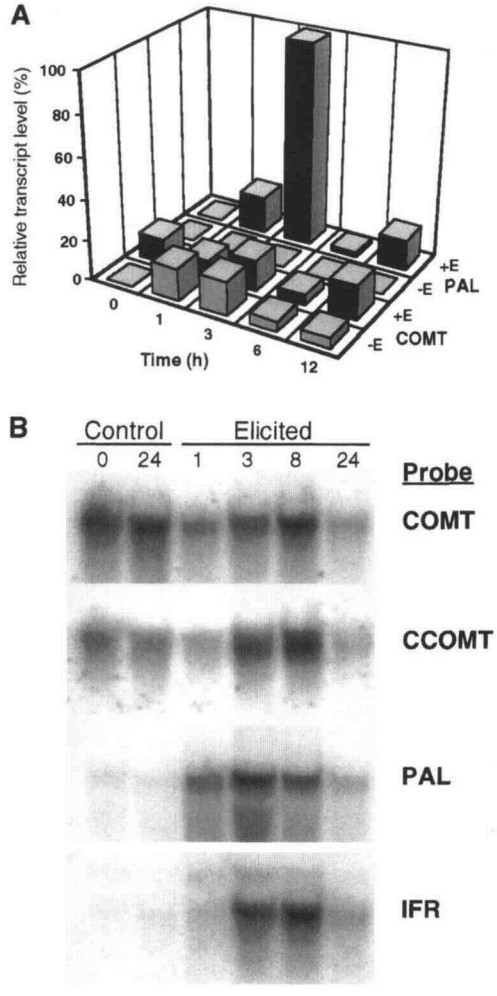

Figure 5. A, Relative levels of COMT and PAL transcripts in polysomal RNA isolated from control (−E) and elicitor-treated (+E) alfalfa cell-suspension cultures (line A1) at the times shown. Autoradiograms of northern blot analyses were scanned with an image analyzer, and values were normalized to the maximum value for PAL as 100%. The value for COMT at time zero minus elicitor was not determined. B, Northern blot analysis of PAL, COMT, CCOMT, and IFR transcripts in the polysomal fraction of elicitor-treated and control cells of line A12. Exposure times are as in Figure 4B.

Table I. *Changes in medicarpin, NDF, Klason lignin, and lignin methoxyl groups in control and elicitor-treated alfalfa cells (line A12)*

Treatment[a]	Time	Medicarpin	NDF	Klason Lignin	Lignin Methoxyl Groups
	h	*μg/g fresh wt*	*%*	*% dry cells*	*% Klason lignin*
−Elicitor	0	0	14.52 ± 1.09[b]	2.45 ± 0.49[b]	1.34 ± 0.16[b]
	4	0	ND[c]	ND	ND
	24	0	13.03 ± 0.92	2.76 ± 0.41	3.41 ± 0.04
+Elicitor	4	6.0 ± 0.3	ND	ND	ND
	8	23.8 ± 2.1	ND	ND	ND
	24	155.1 ± 6.4	20.3 ± 1.91	4.64 ± 0.69	6.41 ± 0.47

[a] Cells were treated with yeast elicitor or water at time zero. [b] All values are averaged; the spread of determinations from duplicate cell batches were treated and processed in parallel. [c] ND, Not determined.

COMT and CCOMT activities, we reassessed these earlier results using more rigorous criteria for the presence of wall-esterified phenolics and/or lignin.

GC-MS and HPLC analysis of material saponified from cell-wall preparations from elicited and control cell cultures failed to detect ferulic acid, sinapic acid, or any other wall-esterified hydroxycinnamic acid derivative (data not shown). Klason lignin was measured after isolation of NDF, a pectin-free cell-wall preparation, and expressed as a percentage of dry cell weight. In cell line A1, which is shown in Figure 6, the values slightly decreased in the elicited cells, then slowly increased to control values by 9 h postelicitation. Kjeldahl nitrogen analysis revealed that a significant proportion (approximately 38%) of the material measured as Klason lignin was most probably proteinaceous. Phloroglucinol (a reagent that detects coniferaldehyde end groups in lignin) stained the alfalfa cells very weakly, and staining was not increased in the elicited cells (data not shown), providing further evidence for a lack of elicitor-induced lignification. The level of lignin as determined by soluble, UV-absorbing material resulting from the thioglycolic acid extraction procedure (Doster and Bostock, 1988) appeared to increase on elicitation of these cells in a manner quantitatively similar to the changes in COMT activity (Fig. 6). However, pyrolysis GC-MS of cell-wall samples from control and elicited cells revealed very low levels of lignin monomeric material (guaicol and 4-ethylguaicol derived from guaiacyl units, with no products derived from syringyl units), and this did not change upon elicitation (Fig. 7). Very low levels of 4-vinylphenol and 4-vinylguiacol, derived from wall-bound 4-coumaric and

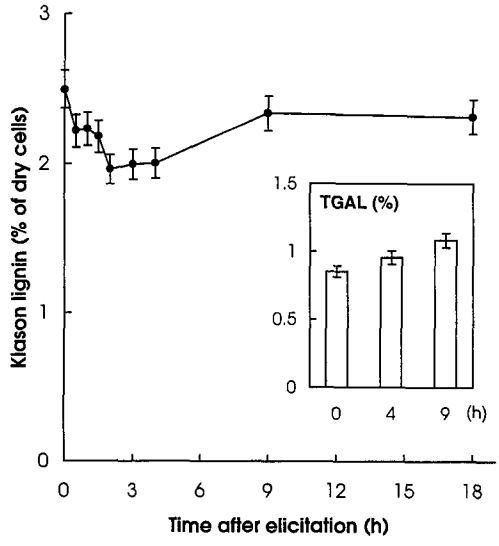

Figure 6. Changes in the levels of Klason lignin as a percentage of dry cell matter in the elicitor-treated alfalfa cell cultures (line A1). Inset, Thioglycolic acid lignin levels as a percentage of dry cell matter in the same elicited cultures.

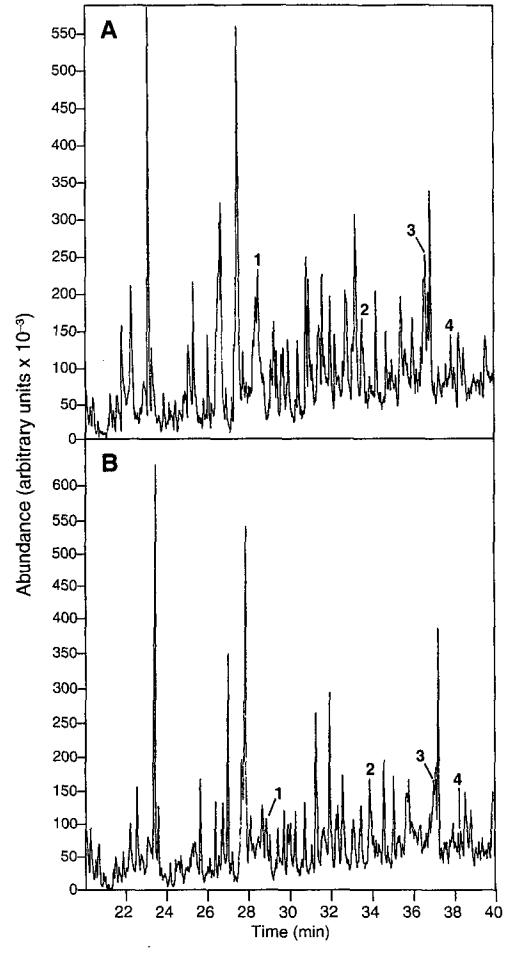

Figure 7. Pyrolysis GC-MS data showing GC traces of pyrolysis products from cell-wall preparations from unelicited (A) and elicited (B) alfalfa suspension cells (line A1). Wall-bound, phenolic-derived products identified by MS analysis were: 1, guaiacol; 2, vinylphenol; 3, ethylguaiacol; and 4, vinylguaiacol.

ferulic acids, respectively, were also detected and also did not increase in response to elicitation (data not shown). There was no indication of sinapyl residues in the alfalfa cell walls. As a control for this technique, pyrolysis GC-MS of cell walls from healthy alfalfa stems revealed high levels of 11 different pyrolysis derivatives from lignin monomers, including the predicted syringyl-derived units (data not shown).

In the A12 cell line, there was a significant increase in both NDF and Klason lignin as a percentage of dry matter in response to elicitation (Table I). Since these cells exhibited no increase in COMT or CCOMT activities, we determined the methoxyl group content of the Klason lignin to ascertain whether there was increased synthesis of guaiacyl and syringyl units (Table I). Unelicited cells had a methoxyl group content of approximately 1.3% of Klason lignin. As the average value for dicot lignin should be about 17%, this analysis also shows that the alfalfa cell walls do not contain significant amounts of guaiacyl/syringyl lignin. There was, however, an increase in methoxyl content on elicitation to over 6%, suggesting that some methoxylated phenylpropane units were being deposited in the alfalfa cell walls in spite of the lack of increase of COMT and CCOMT activity. It should be noted that all of the enzyme activities measured in cell line A12 were higher than those in line A1 (Fig. 2), which might explain the differences in phenolic deposition in the two lines.

Taken together, the above results indicate that, although elicitation may lead to changes in cell-wall composition, structure, or solubility, it does not result in increased synthesis of true lignin in the alfalfa cells. Furthermore, the material detected in elicited cells by thioglycolic acid lignin analysis is probably the aromatic group of immobilized cell-wall proteins (Bradley et al., 1992). Changes in nonphenolic cell-wall polymers have been reported in elicitor-treated bean cell suspensions; however, these also accumulated wall-esterified phenolics (Bolwell et al., 1985).

Although there are reports in the literature of elicitor-induced lignin accumulation in plant cell-suspension cultures (Bruce and West, 1989; de Sá et al., 1992; Boudet et al., 1995), many authors have been careful to stress that the strict criteria needed for the identification of cell-wall phenolic material as "lignin" (namely demonstration of guaiacyl and sometimes syringyl residues with the characteristic linkage patterns) have not been met in their studies (e.g. de Sá et al., 1992). Furthermore, solid-state NMR studies of pine (*Pinus tadea*) cell suspensions growing in a 2,4-D-containing medium have unequivocally indicated that no lignin is present in the primary cell walls (Eberhardt et al., 1993). However, cell cultures of *Pinus banksiana* appear to accumulate true gymnosperm lignin, as assessed by a range of chemical and biochemical criteria, in response to elicitation (Campbell and Ellis, 1992a). In elicited castor bean cells the increase in lignin after 12 h accounted for an extra 2% of dry matter (Bruce and West, 1989), similar to that seen in one of the cell lines we have analyzed. However, the changes we have measured as thioglycolic acid lignin were considerably less than those obtained by other studies that have used this method to determine changes in lignin in elicited cells (Bruce and West, 1989; de Sá et al., 1992).

Conclusion: Control Points for the Selectivity of Elicitor-Mediated Gene Expression

Previous work on the relationship between gene transcription and subsequent metabolic events in elicitor-treated alfalfa cells has demonstrated a correlation between an increased transcription rate and subsequent increases in enzymatic activity for a range of genes involved in the core phenylpropanoid pathway and the flavonoid/isoflavonoid branch pathway (Dixon et al., 1995; Ni et al., 1996). COMT and CCOMT introduce the first methoxy group into the aromatic rings of hydroxycinnamic acids and monolignols, and have been implicated in the biosynthesis of wall-bound phenolic compounds in plant cells (Maule and Ride, 1976; Pakusch et al., 1989; Jaeck et al., 1992). The transcription of their corresponding genes is strongly activated in alfalfa cell cultures in response to an elicitor from yeast cell walls, to a similar or greater extent than that of PAL, the first enzyme of the phenylpropanoid pathway. However, the increase in transcription of COMT and CCOMT does not translate into a significant increase in extractable enzymatic activity. In the case of COMT a significant basal level of transcripts is either unaffected or decreases slightly following elicitation, whereas CCOMT transcripts are induced from very low basal levels, but to a much lower level than PAL transcripts. This suggests that the reason for the lack of increase in enzymatic activity is the failure of the cells to accumulate newly formed COMT or CCOMT transcripts. In neither case does the lack of increase in enzymatic activity appear to result from reduced polysomal recruitment, as the levels of polysomal RNAs encoding COMT and CCOMT directly reflected their total levels, as is also seen for PAL and IFR transcripts. In view of their high transcription rate but low steady-state transcript levels, it is possible that COMT and CCOMT transcripts are less stable than PAL or IFR transcripts following elicitation. It has been reported that a bean cell-wall Pro-rich protein transcript is destabilized in response to elicitation (Zhang et al., 1993), although in contrast with the present results, its transcription rate remained constant.

Since elicitation induces COMT and CCOMT transcription but does not result in increased enzymatic activities that lead to increased product accumulation, de novo gene transcription alone does not determine the pattern of metabolism in elicited alfalfa cells. Our study points to a need for further work on posttranscriptional control mechanisms, including mRNA stability studies, in the area of plant phenylpropanoid biosynthesis. Our results also provide a warning against relying on the measurement of promoter activity or transcript levels as indicators of the metabolic status of plant cells.

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LITERATURE CITED

Blount JW, Dixon RA, Paiva NL (1993) Stress responses in alfalfa (*Medicago sativa* L.). XVI. Antifungal activity of medicarpin and its biosynthetic precursors: implications for the genetic manipulation of stress metabolites. Physiol Mol Plant Pathol **41:** 333–349

Bolwell GP, Robbins MP, Dixon RA (1985) Metabolic changes in elicitor-treated bean cells: enzymic responses in relation to rapid changes in cell wall composition. Eur J Biochem **148:** 571–578

Boudet AM, Lapierre C, Grima-Pettenati J (1995) Biochemistry and molecular biology of lignification. New Phytol **129:** 203–236

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell **70:** 21–30

Bruce RJ, West CA (1989) Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. Plant Physiol **91:** 889–897

Campbell MM, Ellis BE (1992a) Fungal elicitor-mediated responses in pine cell cultures: cell wall-bound phenolics. Phytochemistry **31:** 737–742

Campbell MM, Ellis BE (1992b) Fungal elicitor-mediated responses in pine cell cultures. I. Induction of phenylpropanoid metabolism. Planta **186:** 409–417

Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem **162:** 156–159

Collinge DB, Slusarenko AJ (1987) Plant gene expression in response to pathogens. Plant Mol Biol **9:** 389–410

Dalkin K, Edwards R, Edington B, Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.). I. Elicitor-induction of phenylpropanoid biosynthesis and hydrolytic enzymes in cell suspension cultures. Plant Physiol **92:** 440–446

DeLisle AL, Crouch ML (1989) Seed storage protein transcription and mRNA levels in *Brassica napus* during development and in response to exogenous abscisic acid. Plant Physiol **91:** 617–623

de Sá MM, Subramaniam R, Williams FE, Douglas CJ (1992) Rapid activation of phenylpropanoid metabolism in elicitor-treated hybrid poplar (*Populus trichocarpa* Torr. & Gray × *Populus deltoides* Marsh) suspension-culture cells. Plant Physiol **98:** 728–737

Dixon RA, Harrison MJ (1990) Activation, structure and organization of genes involved in microbial defense in plants. Adv Genet **28:** 165–234

Dixon RA, Harrison MJ, Paiva NL (1995) The isoflavonoid phytoalexin pathway: from enzymes to genes to transcription factors. Physiol Plant **93:** 385–392

Doster MA, Bostock RM (1988) Quantification of lignin formation in almond bark in response to wounding and infection by *Phytophthora* species. Phytopathology **78:** 473–477

Eberhardt TL, Bernards MA, He L, Davin LB, Wooten JB, Lewis NG (1993) Lignification in cell suspension culture of *Pinus taeda*. J Biol Chem **268:** 21088–21096

Fahrendorf T, Ni W, Shorrosh BS, Dixon RA (1995) Stress responses in alfalfa (*Medicago sativa* L.). XIX. Transcriptional activation of oxidative pentose phosphate pathway genes at the onset of the isoflavonoid phytoalexin response. Plant Mol Biol **28:** 885–900

Gowri G, Bugos RC, Campbell WH, Maxwell CA, Dixon RA (1991a) Stress responses in alfalfa (*Medicago sativa* L.). X. Molecular cloning and expression of *S*-adenosyl-L-methionine:caffeic acid 3-O-methyltransferase, a key enzyme of lignin biosynthesis. Plant Physiol **97:** 7–14

Gowri G, Paiva NL, Dixon RA (1991b) Stress responses in alfalfa (*Medicago sativa* L.). XII. Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell cultures and developing plants. Plant Mol Biol **17:** 415–429

Jaeck E, Dumas B, Geoffroy P, Favet N, Inzé D, Van Montagu M, Fritig B, Legrand M (1992) Regulation of enzymes involved in lignin biosynthesis: induction of O-methyltransferase mRNAs during the hypersensitive reaction of tobacco to tobacco mosaic virus. Mol Plant-Microbe Interact **5:** 294–300

Jorrin J, Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.). II. Purification, characterization and induction of phenylalanine ammonia-lyase isoforms from elicitor treated cell suspension cultures. Plant Physiol **92:** 447–455

Kaar WE, Cool LG, Merriman MM, Brink DL (1991) The complete analysis of wood polysaccharides using HPLC. J Wood Chem Technol **11:** 447–463

Kodrzycki R, Boston RS, Larkins BA (1989) The opaque-2 mutation of maize differentially reduces zein gene transcription. Plant Cell **1:** 105–114

Kühnl T, Koch Y, Heller W, Wellmann E (1989) Elicitor induced S-adenosyl-L-methionine:caffeoyl-CoA 3-O-methyltransferase from carrot cell suspension cultures. Plant Sci **60:** 221–225

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol **41:** 455–496

Maule AJ, Ride JP (1976) Ammonia-lyase and O-methyl transferase activities related to lignification in wheat leaves infected with *Botrytis*. Phytochemistry **15:** 1661–1664

Maxwell CA, Harrison MJ, Dixon RA (1993) Molecular characterization and expression of alfalfa isoliquiritigenin 2′-O-methyltransferase, an enzyme specifically involved in the biosynthesis of an inducer of *Rhizobium meliloti* nodulation genes. Plant J **4:** 971–981

Mignery GA, Pikaard CS, Hannapel DJ, Park WD (1984) Isolation and sequence analysis of cDNAs for the major potato tuber protein, patatin. Nucleic Acids Res **12:** 7987–8000

Ni W, Fahrendorf T, Ballance GM, Lamb CJ, Dixon RA (1996) Stress responses in alfalfa (*Medicago sativa* L.). XX. Transcriptional activation of phenylpropanoid pathway genes in elicitor-induced cell suspension cultures. Plant Mol Biol **30:** 427–438

Ni W, Paiva NL, Dixon RA (1994) Reduced lignin in transgenic plants containing an engineered caffeic acid O-methyltransferase antisense gene. Transgenic Research **3:** 120–126

Orr JD, Edwards R, Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.). XIV. Changes in the levels of phenylpropanoid pathway intermediates in relation to regulation of L-phenylalanine ammonia-lyase in elicitor treated cell suspension cultures. Plant Physiol **101:** 847–856

Paiva NL, Edwards R, Sun Y, Hrazdina G, Dixon RA (1991) Stress responses in alfalfa (*Medicago sativa* L.). XI. Molecular cloning and expression of alfalfa isoflavone reductase, a key enzyme of isoflavonoid phytoalexin biosynthesis. Plant Mol Biol **17:** 653–667

Pakusch A-E, Kneusel RD, Matern U (1989) S-Adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-O-methyltransferase from elicitor-treated parsley cell suspension cultures. Arch Biochem Biophys **271:** 488–494

Pearce RB, Ride JP (1982) Chitin and related compounds as elicitors of the lignification response in wounded wheat leaves. Physiol Plant Pathol **20:** 119–123

Ralph J, Hatfield RD (1991) Pyrolysis-GC-MS characterization of forage materials. J Food Agric Chem **39:** 1426–1437

Robertsen B (1986) Elicitors of the production of lignin-like compounds in cucumber hypocotyls. Physiol Mol Plant Pathol **28:** 137–148

Robertson JB, Van Soest PJ (1980) The detergent system of analysis and its application to human foods. *In* WPT James, O Theander, eds, The Analysis of Dietary Fiber in Food. Marcel Dekker, New York, p 123

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shorrosh BS, Dixon RA, Ohlrogge JB (1994) Molecular cloning, characterization and elicitation of acetyl CoA carboxylase from alfalfa. Proc Natl Acad Sci USA **91:** 4323–4327

Stöckigt J, Zenk MH (1975) Chemical synthesis and properties of hydroxycinnamoyl-coenzyme A derivatives. Z Naturforsch **30c:** 352–358

TAPPI (1972) Methoxyl Content of Pulp and Wood: TAPPI Standard Method 209 su-72. Technical Association of the Pulp and Paper Industry (TAPPI), Atlanta, GA

Vance CP, Kirk TK, Sherwood RT (1980) Lignification as a mechanism of disease resistance. Annu Rev Phytopathol **18:** 259–288

Ye Z-H, Kneusel RE, Matern U, Varner JE (1994) An alternative methylation pathway in lignin biosynthesis in *Zinnia*. Plant Cell **6:** 1427–1439

Zhang S, Sheng J, Liu Y, Mehdy MC (1993) Fungal elicitor-induced bean proline-rich protein mRNA down-regulation is due to destabilization that is transcription and translation dependent. Plant Cell **5:** 1089–1099