Plant Cell Reports (1989) 8:504–507

Plant Cell Reports

Defense gene expression in elicitor-treated cell suspension cultures of french bean cv. Imuna

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Received July 28, 1989/Revised version received October 3, 1989 – Communicated by J. M. Widholm

ABSTRACT.

Cell suspension cultures of bean (Phaseolus vulgaris) cv. Imuna accumulated isoflavonoid phytoalexins on exposure to elicitor from the phytopathogenic fungus Colletotrichum lindemuthianum (CL). This was preceeded by rapid increases in the activities of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS). However, the patterns of expression of PAL and CHS genes differed from those observed in cultures of a previously studied bean cultivar. The relative levels of transcripts from individual members of the CHS multigene family differed significantly at 1.5 h compared to 22.5 h after elicitation. More strikingly, three PAL genes were expressed in cultivar Imuna in response to fungal elicitor, whereas two are expressed in elicitor-treated cell cultures of cultivar Canadian Wonder.

INTRODUCTION.

PAL and CHS catalyze key reactions in the biosynthesis of the isoflavonoid phytoalexins of the Leguminosae. These are, respectively, the first committed step in the biosynthesis of phenylpropanoid secondary products from L-phenylalanine and the first reaction of the branch pathway leading to the formation of flavonoids and isoflavonoids. In suspension cultures of bean (Robbins et al. 1985), soybean (Ebel et al. 1984), chickpea (Barz et al. 1989) and alfalfa (Dalkin et al. 1989), isoflavonoid accumulation in response to elicitors is preceeded by rapid transient increases in the extractable activities of these and other phytoalexin biosynthetic enzymes. In bean and soybean, this has been shown to result from increased translatable activities and/or steady state levels of the respective mRNAs (Lawton et al. 1983, Ebel et al. 1984). Both PAL and CHS are encoded by multigene families in bean (Cramer et al. 1989, Ryder et al. 1987), their induction in response to various environmental stimuli being associated with differential expression of individual members of the family. In suspension cultures of cultivar Canadian Wonder, transcripts from at least four CHS genes are coordinately induced in response to elicitor, whereas two of the three PAL genes are elicitor-inducible (Ryder et al. 1987, Cramer et al. 1989). We now report different patterns of expression of PAL and CHS transcripts in suspension cultures of cultivar Imuna, which accumulates higher levels of isoflavonoid phytoalexins than cultivar Canadian Wonder.

MATERIALS AND METHODS.

Elicitation of cell suspension cultures. Cell suspension cultures from cultivars Imuna and Canadian Wonder, derived identically from root explant callus of the same age (at least 15 passages after initiation) were grown in a modified Schenk and Hildebrandt medium as described (Dixon et al. 1981). They were exposed to elicitor from the cell walls of CL (60 µg glucose equivalents/ml culture, Dixon and Lamb 1979), 6 days after subculture into fresh medium. Cells were filtered, frozen with liquid nitrogen and stored at -70°C.

Analysis of phytoalexins. Cells were extracted in ethanol (50 ml/g fresh weight) and extracts worked up for HPLC analysis on a Partisil 10 ODS 1 reverse phase column as described (Dixon et al. 1989). The eluting solutions were 15% acetic acid and acetonitrile with a gradient of acetonitrile from 10% to 55% in 90 min, then to 100% acetonitrile after 100 min. The eluant was monitored at 280 nm. Compounds were identified by UV spectroscopy and co-chromatography on HPLC and TLC with authentic standards.

Enzyme assays. PAL and CHS activities were determined in buffered extracts of elicitor-treated cells as described previously (Robbins et al. 1985).

Isolation and translation in vitro of polysomal mRNA. Polysomal mRNA was isolated from cell cultures and translated in vitro in an mRNA-dependent rabbit reticulocyte lysate translation system as described previously (Hamdan and Dixon 1987). CHS subunits were immunoprecipitated with anti-(parsley CHS) serum and protein A Sepharose as described (Lawton et al. 1983).

Plasmid and M13 probes. The bean CHS subclone pRP1-3 contained a portion of the 3'-untranslated region of CHS 1 in the riboprobe vector pSP65; this insert sequence was highly specific for CHS 1 mRNAs. Similarly, pRP4-3 contained a gene-specific 76 bp fragment (in pSP65) from the 3'-untranslated region of CHS 4 (Ryder et al. 1987). pRP4-5 was a subclone of the 5'-coding region of CHS 4 in pSP64, and the insert was homologous to all bean CHS sequences. pRP17 contained a full-length cDNA from CHS 17 (Ryder et al. 1987) in the antisense orientation in pSP65.

A bean PAL1-specific subclone contained a BglII-PstI fragment from the 3'-end of pPAL1 cDNA (Edwards et al. 1985) in M13 mp8 (Fig. 1). Similar gene-specific subclones were used for the detection of transcripts from PAL2 (a BglII-EcoR1 fragment from the 3'-end of genomic clone gPAL2 in M13mp9)

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and PAL3 (a 1182 bp HindIII fragment spanning the intron of genomic clone gPAL3 in M13mp8) (Cramer et al. 1989). Highly labelled single strand gene-specific probes were synthesized from the M13 subclones with Klenow fragment after initiating with M13 universal primer; probes were then cut with SacI (PAL1, yielding a 235 bp fragment), SnaBI (PAL2, yielding a 129 bp fragment) or EcoRI (PAL3, yielding an approximately 1 kb fragment). Probes were purified on a 6% sequencing gel, ethanol-precipitated and taken up in hybrid select buffer (Mason and Williams 1985) for hybridization to mRNA samples and analysis by S1 protection.

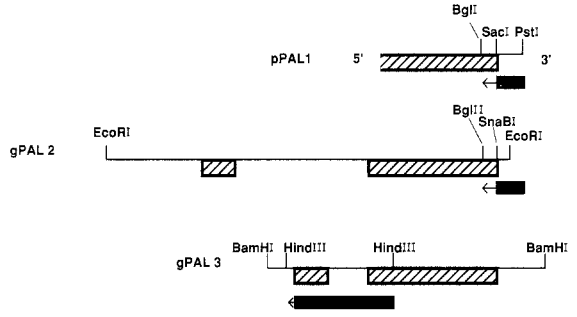

Figure 1. Origin of the gene specific probes for measurement of PAL transcript levels (see text for details). Hatched areas represent exons; the arrows delineate the subcloned sequences, the filled in boxes the actual probes.

S1 nuclease and RNase protection assays. For S1 nuclease protection, polysomal mRNA (12 µg) was incubated overnight at 50°C with 10 µl labelled probe (2 x 10^4 cpm) in hybrid select buffer. Tubes were quench cooled to 4°C, and 200 µl ice cold 1 x S1 buffer (30mM sodium acetate pH 4.5, 250 mM NaCl, 1mM ZnSO4, 5% (v/v) glycerol) added, followed by 250 units S1 nuclease. After incubation for 1 h at 37°C, samples were phenol-extracted, ethanol precipitated (after addition of 10 µg tRNA) and taken up in 4 µl of Tris-EDTA buffer, pH 8.0. Samples were analysed on 35 cm, 6% sequencing gels along with Mr markers (labelled HinfI-digested pBR322 plasmid). RNase protection assays with riboprobe from pRP4-5 were performed as described (Liang et al. 1989).

Blot hybridization analyses. mRNA samples were transferred to PALL Biodyne nylon membranes using a Bio-Rad dot blot apparatus. Blots were hybridized overnight in a solution containing 50% formamide at 60°C with labelled CHS riboprobes according to the PALL Biodyne protocol for RNA blots. Blots were washed four times in 2 x SSC, 0.1% SDS for 5 min at room temperature, followed by two washes in 0.1 x SSC, 0.1% SDS for 15 min at 50°C. Autoradiography was for 2 days at -70°C.

RESULTS AND DISCUSSION.

Phytoalexin accumulation. Elicitor-treated cell cultures of bean cv. Imuna accumulated the isoflavonoid phytoalexins phaseollin and kievitone, with no corresponding accumulation of isoflavonoid precursors of these compounds (Fig. 2). A major unidentified metabolite of retention time 41 min rapidly disappeared on exposure of cells to elicitor. At 22.5 h post-elicitation, kievitone was the major phytoalexin present (135 nmol/g fresh wt), whereas phaseollin began to predominate after 50 h. In previous experiments with an earlier Imuna cell line (Robbins et al. 1985), kievitone accumulation similarly preceeded that of phaseollin, although the response was more rapid.

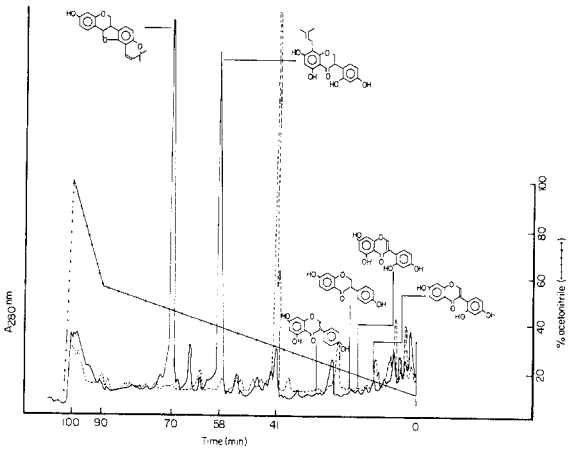

Figure 2. HPLC analysis of isoflavonoids from cultured bean cells. Profiles show material from unelicited cells (dashed line) or from cells exposed to elicitor for 22.5 h (solid line). ⟷ = acetonitrile gradient. The identified compounds and retention times are: 2'-hydroxydaidzein (12 min); 2'-hydroxygenistein (17 min); daidzein (19 min); genistein (29 min); kievitone (58 min); phaseollin (73 min).

Changes in CHS gene expression. Polysomal mRNA was isolated from cell cultures 1.5 h and 22.5 h after addition of elicitor; these times were chosen as being approximate early and late maxima for appearance of CHS transcripts based on previous observations in elicitor-treated cultures of bean cultivar Canadian Wonder (Lawton et al. 1983, Ryder et al. 1984, Robbins et al. 1985). In vitro translation followed by specific immunoprecipitation of CHS subunits revealed slightly lower total translatable CHS mRNA activity at 22.5 h than at 1.5 h (Fig. 3) which correlated approximately with the CHS enzyme activities measured subsequent to these two times (Fig. 4)

Total CHS transcript levels in the polysomal fraction measured by dot blot analysis using two gene-non-specific riboprobes (from pRP4-5 and pRP17) were approximately equal at 1.5 h and 22.5 h post-elicitation (Fig. 3B). However, transcripts from the CHS gene 1 (detected with the gene-specific riboprobe from pRP1-3) were present at a much higher level 22.5 h post elicitation than at 1.5 h post elicitation (Fig. 3B). As the steady-state level of total CHS transcripts (and gene 4 transcripts detected with riboprobe from pRP4-3, data not shown) was similar at 1.5 h and 22.5 h, the major proportion of the CHS activity at 1.5 h post-elicitation must be due to transcripts from CHS 4 and other members of the CHS multigene family.

The rapid initial increase in CHS enzyme activity in response to elicitor was preceeded by an increase in total CHS transcripts reaching maximum levels 4 h post-elicitation (Fig. 4). In contrast, transcripts specific for the CHS 1 gene increased very slowly in response to elicitor, only reaching a maximum after 24 h. In cell cultures of cultivar Canadian Wonder treated with an identical elicitor preparation a more closely co-ordinated induction of different CHS transcripts was observed and no member of the multigene family gave rise to higher transcript levels between 10-30 h than observed at the peak 4 h post-elicitation (Ryder et al. 1987). The elevated activities of CHS from 24 to 36 h in Imuna cultures preceed the major phase of

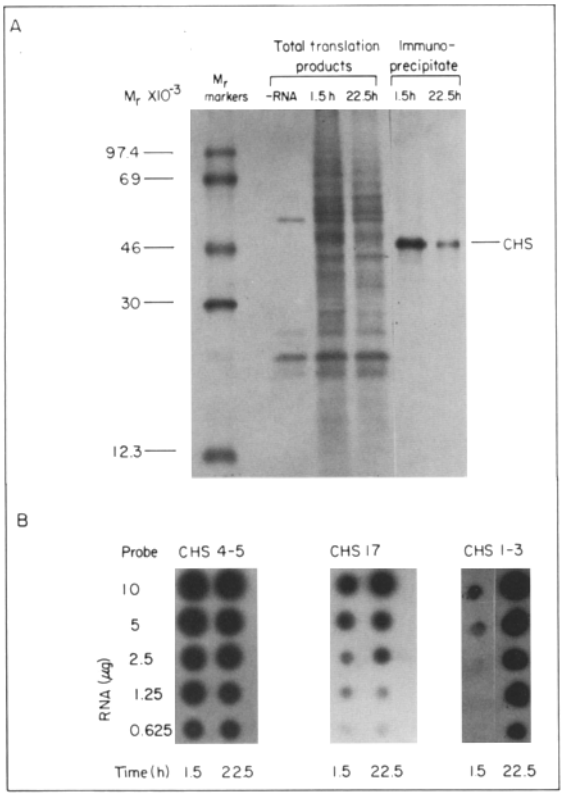

Figure 3. Analysis of CHS mRNA in the polysomal fraction from cells exposed to elicitor for 1.5 h and 22.5 h. **A.** In vitro translation followed by SDS-PAGE analysis of total translation products and polypeptides immunoprecipitated with anti-(parsley CHS) serum and protein A Sepharose. **B.** RNA dot blots probed with labelled riboprobes complementary to the 5'-end of CHS 4, CHS 17 cDNA and the 3'-end of CHS 1.

accumulation of the 5-deoxy-isoflavonoid-derived phytoalexin phaseollin, whose synthesis requires the activity of a "deoxy-chalcone synthase" which may nevertheless utilize the "normal" chalcone synthase as condensing enzyme (Dewick et al. 1982, Welle and Grisebach 1988). It is therefore possible that CHS 1 is specifically involved in phaseollin biosynthesis, although the very strong coding sequence homologies between the different CHS transcripts (Ryder et al. 1987) do not immediately suggest functional distinctions.

Changes in PAL gene expression. Three distinct PAL genes have been characterized in bean (Cramer et al. 1989). PALs 1, 2 and 3 are present in the genome of cultivar Canadian Wonder, and all are expressed under the appropriate environmental/-inducing conditions (Liang et al. 1989). S1 nuclease protection assays using probes specific for transcripts from each of these genes revealed that expression of all three was activated by elicitor treatment in the Imuna cultures (Fig. 5), although expression of PAL2 was weak and was not observed in subsequent experiments with later subcultures of the same culture line. PAL2 was, however, strongly induced in newly initiated suspensions and this inducibility has now been observed over at least 15 passages, indicating that the loss of expression of PAL 2 is a relatively slow epigenetic/physiological phenomenon. Expression of PAL3, here confirmed by the two S1-protected fragments resulting from removal of the intron sequence from the labelled probe

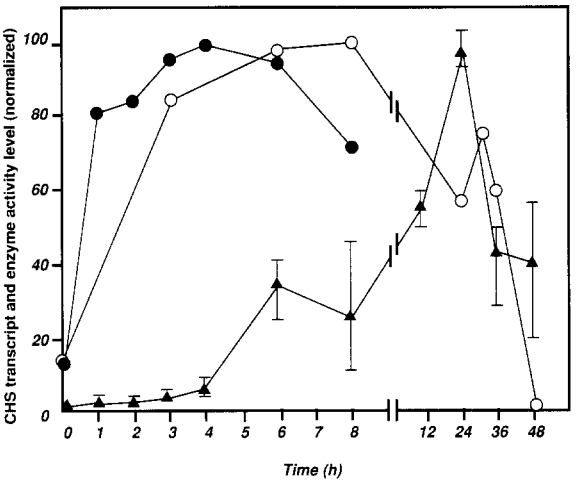

Figure 4. Time course for appearance of CHS enzyme activity (O-O), CHS gene 1 transcripts (▲-▲) and total CHS transcripts (● - ●) in elicitor-induced Imuna cell cultures. CHS-1 transcript levels are calculated from densitometric analysis of RNA dot blots (data normalized relative to highest value (100%), and indicating spread of values from 3 determinations). Total transcript levels were measured by RNase protection.

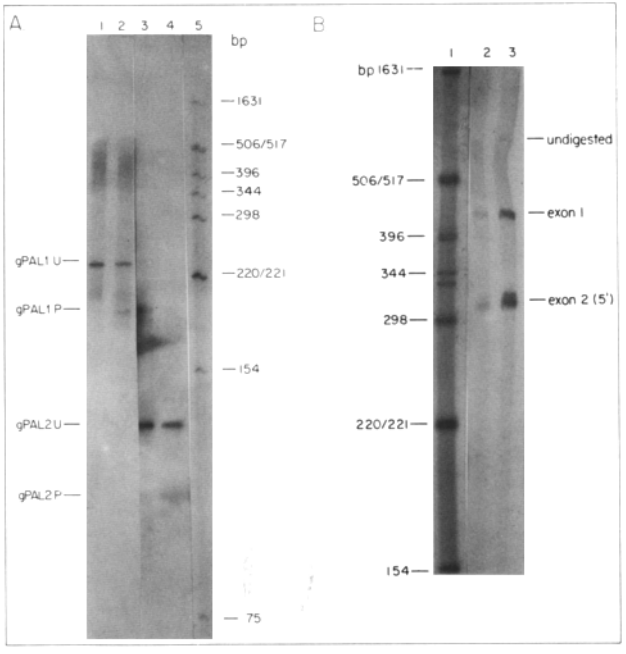

Figure 5. **A.** S1 protection analysis of the steady-state levels of PAL1 and PAL2 transcripts in the polysomal RNA fraction of unelicited bean cell cultures (tracks 1 and 3) and cultures exposed for 3h to Colletotrichum elicitor (tracks 2 and 4). Transcripts were measured as fragments protected against S1 nuclease digestion by single strand probes specific for PAL1 (tracks 1 and 2) and PAL2 (tracks 3 and 4). Track 5 = Hinf1-digested pBR322 Mr markers. U = undigested probe, P = protected fragment. **B.** S1 protection analysis of PAL 3 transcripts. Track 1 = Mr markers, track 2 = RNA from unelicited cells, track 3 = RNA from cells exposed to Colletotrichum elicitor for 3h.

(Fig. 5B), was not observed in previous studies using elicitor-induced cultures of cultivars Canadian Wonder or Tendergreen, in which PAL2 is strongly induced (Cramer et al. 1989; Liang et al. 1989).

Levels of PAL1 and PAL3 transcripts increased very rapidly in elicited Imuna cultures, reaching maxima at 2-3 h after exposure to elicitor (Fig. 6A). These findings have been reproduced in a number of subsequent experiments with this and other Imuna cell lines, in which initial maxima in the levels of PAL 1,2 and 3 transcripts were observed at 1-3, 4-6 and 2-3 hours post elicitation respectively. A late increase in PAL3 transcripts was also observed at 48h post-elicitation.

As we had observed a loss of elicitor-induced PAL2 expression with increasing passages of the Imuna suspensions, it was possible that PAL3 expression in cultivar Canadian Wonder was similarly related to time in liquid culture. We therefore established new suspensions of Canadian Wonder from callus stocks of a similar age to those used for initiation of the Imuna suspensions; S1 protection analysis confirmed the lack of expression of PAL3 in these cultures (Fig. 6B).

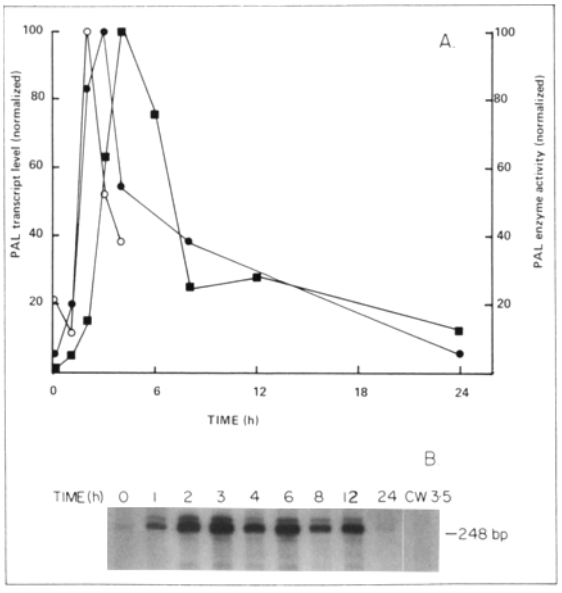

Figure 6 **A.** Time course for the appearance of PAL enzyme activity (■-■) and PAL1 (○-○) and PAL3 (●-●) transcripts in elicitor-treated cell cultures. Data for transcript levels obtained by densitometric analysis of autoradiographs from S1-protection experiments. Maximum PAL activity (100 arbitrary units) = 40 μkat/kg protein. **B.** Autoradiogram showing the S1-protected 5' second exon fragment of PAL3 in a separate experiment. The track labelled CW3.5 shows the lack of PAL 3 transcript in cultures of cultivar Canadian Wonder 3.5 h post-elicitation.

Conclusions. The CHS activity in cultures of cultivar Imuna at late times post-elicitation is associated with a pattern of transcripts from the CHS multigene family very different from that observed within the first few hours, unlike the case in Canadian Wonder cultures growing in the same medium. PAL3 transcripts are strongly induced by elicitor in Imuna, but although present, this gene is not activated in response to elicitor in cultures of cultivar Canadian Wonder used in this and previous work. We have not yet ruled out the possibility that a rapid loss of ability to express PAL3 occurs during early passages of Canadian Wonder callus. If so, this would represent a rapid epigenetic change which we have never observed in Imuna cells cultured under identical conditions. Our data indicate that cultivar-dependent differences may exist in the ability to express iso-genes in response to a particular external stimulus in cultured cells, and it may therefore be unwise to refer to a gene as elicitor-inducible or non-inducible on the basis of experiments with a single cell culture line.

ACKNOWLEDGEMENTS. We thank Tom Ryder and Keith Edwards for construction of gene-specific subclones, Klaus Hahlbrock for the gift of anti-(parsley CHS) serum, Chris Gerrish for technical assistance and Scotty McGill for preparing the manuscript. This work was supported by a grant from the United Kingdom Agricultural and Food Research Council to RAD.

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