

Timing of Changes in Protein Synthesis Pattern in Elicitor-Treated Cell Suspension Cultures of Parsley (*Petroselinum crispum*)

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The timing of changes in the protein synthesis pattern of elicitor-treated, [³⁵S]methionine-labelled parsley cells (*Petroselinum crispum*) was analyzed by two-dimensional gel electrophoresis. Five groups were distinguished from a large number of elicitor-responsive as well as unresponsive proteins. Two groups were synthesized *de novo* either early or late after elicitor application; two other groups were strongly reduced in their rates of synthesis either early or late after elicitor application; and one group was not appreciably affected at all. The elicitor-induced changes altered the total protein composition considerably. A few selected, induced proteins were functionally identified. These included two early induced enzymes, phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL), and a late induced enzyme, a bergapton O-methyltransferase (BMT) which is specifically involved in the biosynthesis of furanocoumarin phytoalexins. The biological significance of the observed differential timing of changes in protein synthesis rates is discussed.

Introduction

The response of suspension-cultured parsley cells to treatment with fungal elicitor [1, 2] closely mimics the response of intact parsley leaves to infection with the live fungus *Phytophthora megasperma* f. sp. *glycinea* [3–6]. The defense response of plants to pathogen attack is a concerted action of a variety of biochemical reactions including, in parsley, the synthesis of furanocoumarin phytoalexins [7].

The biosynthesis of furanocoumarin phytoalexins [1, 8] as well as various other effects of elicitor application [9] have been studied extensively in cultured parsley cells. By analyzing several representative genes, it has been shown that the induced accumulation of furanocoumarins and other phenylpropanoid derivatives is regulated at the level of transcription [10–13]. Transcriptional activation of further, functionally unidentified genes has

been demonstrated at the level of nuclear run-off transcripts or cDNAs derived from these transcripts by differential hybridization [6, 14].

In an attempt to more fully describe the response of cultured parsley cells to treatment with elicitor, this paper describes the mRNA translational activity *in vivo* as an indication of differential changes in the gene expression pattern. The translation products were pulse-labelled with [³⁵S]methionine *in vivo* at different times before and after elicitor application and were analyzed on two-dimensional gels.

Materials and Methods

Cell cultures and elicitor treatment

Parsley cells (*Petroselinum crispum* L.) were cultured and propagated as described [15]. Heat-released fungal elicitor was prepared and applied according to published methods [2, 16].

Labelling of proteins *in vivo*

The proteins synthesized 1 h before harvest were pulse-labelled by adding 3.7×10^8 Bq L-[³⁵S]methionine per ml of medium. Cells were extracted with 0.15 mol·l⁻¹ NaCl, 4 mmol·l⁻¹ methionine, 1% Triton X-100, 50 mmol·l⁻¹ Tris-HCl, pH 7.5. Equal amounts of radioactivity were applied to all gels.

Abbreviations: BMT, S-adenosyl-L-methionine: bergapton O-methyltransferase; 4CL, 4-coumarate:CoA ligase; IEF, isoelectric focussing; PAL, phenylalanine ammonia-lyase; PR, pathogenesis-related; SDS-PAGE, sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis.

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Two-dimensional gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis was carried out as described [17]. The isoelectric focussing gel contained a 1:2:2 mixture of pH 3.5–10, pH 4–6 and pH 6–8 ampholines. Separation in the second dimension was in a 10–18% polyacrylamide gradient (SDS-PAGE). Fluorography was as described [18].

Silver staining

Gels were stained with silver nitrate as described [19]. The staining solution had a volume of 300 ml.

Immunoprecipitation

Indirect immunoprecipitation using protein-A-sepharose was performed according to [15]. The origin of antibodies was as described previously [10, 20].

Immunoblot analysis

After gel-electrophoretic separation, the proteins were electrotransferred overnight onto a nitrocellulose membrane [21]. Filters were incubated for 3 h with a 1:200 diluted rabbit antiserum raised against BMT [20] and stained using peroxidase-conjugated *anti*-rabbit IgG.

Quantification of fluorograms

Proteins of interest were scanned, depending on spot size, four to six times in parallel lines, 0.75 mm apart, using a LKB laser densitometer. Densitometric readings were integrated using an Apple IIe minicomputer.

Results

Elicitor-induced changes in protein synthesis

Changes in the synthesis pattern of all *in vivo* pulse-labelled, neutral and acidic soluble proteins upon treatment of cultured parsley cells with elicitor were determined by two-dimensional gel electrophoresis (Fig. 1). As a control, the proteins from untreated parsley cells are shown in Fig. 1 A. Treatment with 10 or 100 $\mu\text{g} \cdot \text{ml}^{-1}$ elicitor 3 h prior to harvest of the cells resulted in the patterns shown in Fig. 1 B and C, respectively. In all cases, [^{35}S]methionine was given to the cells 2 h after ap-

plication of elicitor, 1 h before harvest. Differences between treated and untreated cells were more pronounced with the high dose (100 $\mu\text{g} \cdot \text{ml}^{-1}$) of elicitor, but were otherwise essentially the same as observed with the lower dose (10 $\mu\text{g} \cdot \text{ml}^{-1}$).

Two types of change were noted. On the one hand, at least 25 distinguishable new proteins appeared (arrows in Fig. 1 D); most easily detectable was the appearance of a group of previously identified small and acidic "pathogenesis-related" (PR) proteins [14]. On the other hand, the rate of synthesis of 10 or more other proteins decreased drastically (circles in Fig. 1 D). By comparison, more than 90 constitutively expressed proteins could be distinguished, giving a ratio of clearly identifiable elicitor-induced, or repressed, to constitutively synthesized proteins of approximately 35 to 90, or 39%, a figure indicative of major changes in gene expression.

Two sets of protein spots appeared at previously identified positions [9] and were further analyzed by indirect immunoprecipitation. One series of 7–12 polypeptides, very similar in relative molecular mass, but varying in isoelectric point from pH 5.4–6.4, was identified with an antiserum specific for PAL (Fig. 2 A and B). With antiserum against 4CL, two proteins of nearly identical molecular mass, but with slightly different isoelectric points, were detected (Fig. 2 C and D), as predicted from the recent identification of two 4CL genes and the encoded proteins [22].

Timing of changes

All of the following results are shown for an elicitor concentration of 10 $\mu\text{g} \cdot \text{ml}^{-1}$ culture medium. Higher doses reduced the incorporation of [^{35}S]methionine into protein at late time points to such an extent that analysis of the gel patterns was difficult. Special care was taken to allow for virtually identical conditions of protein labelling, extraction and separation of all samples.

The timing of changes in protein synthesis after elicitor application was followed for 30 h on two-dimensional gels (Fig. 3). We observed five major types of response with regard to timing. To quantitate the time courses, several spots (Fig. 4) representing selected polypeptides of each group were scanned with a laser densitometer and the apparent rates of synthesis were plotted *versus* time.

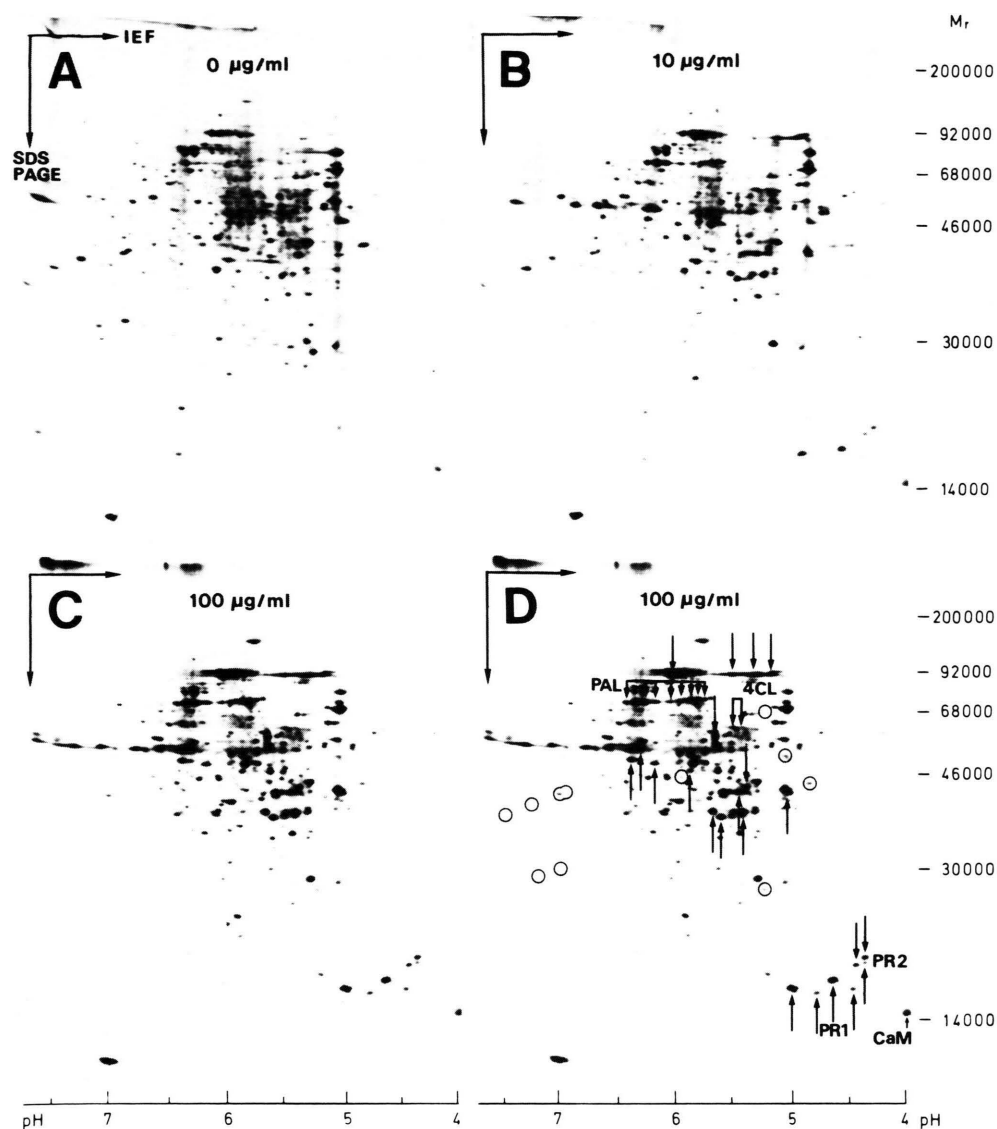
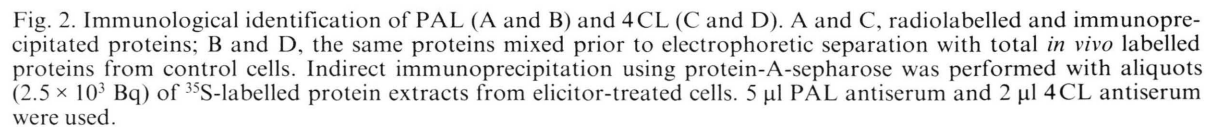


Fig. 1. Two-dimensional gel analysis of *in vitro* pulse-labelled polypeptides from elicitor-treated parsley cells. [^{35}S]Methionine was added between the second and third hour after elicitor application. A, untreated control; B, 10 $\mu\text{g}\cdot\text{ml}^{-1}$ elicitor; C, 100 $\mu\text{g}\cdot\text{ml}^{-1}$ elicitor; D, same as C with arrows indicating newly appearing and circles indicating disappearing polypeptides. PR 1 and PR 2 had been identified previously [14]. Calmodulin (CaM) was localized on the gel by its characteristic mobility shift in the absence of Ca^{2+} [28].



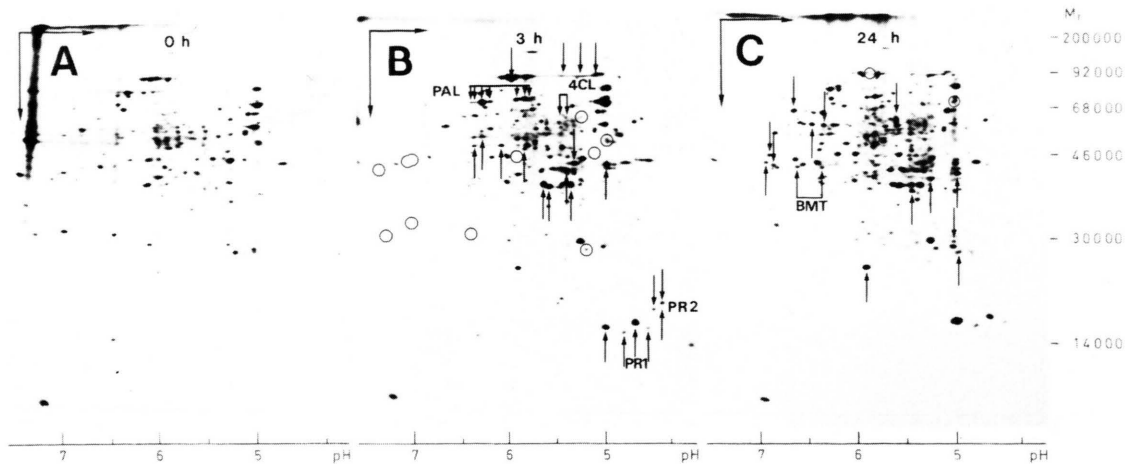


Fig. 3. Early and late changes in protein synthesis after elicitor application ($10 \mu\text{g} \cdot \text{ml}^{-1}$). A, untreated control; B, 3 h after addition of elicitor; C, 24 h after addition of elicitor. The polypeptides were *in vivo* labelled with [^{35}S]methionine 1 h before harvest. Early (B) and late (C) responding polypeptides are indicated either with arrows (increased rates of synthesis) or circles (decreased rates of synthesis).

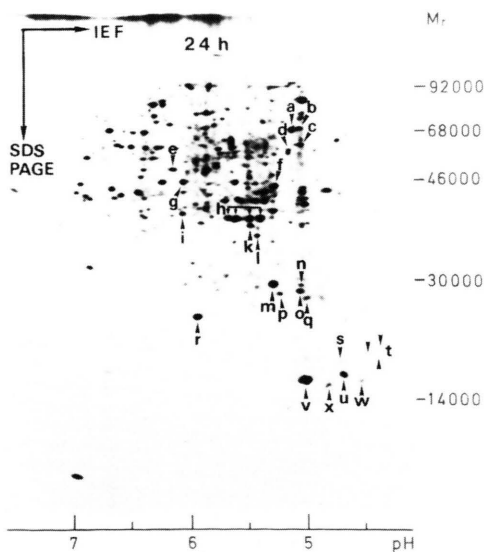


Fig. 4. Polypeptides (a–x) selected for determination of time courses of synthesis (see below, Fig. 5, 6 and 7). The fluorograph shows ^{35}S -labelled proteins synthesized 24 h after administration of elicitor. Arrowheads and letters mark selected proteins. Note that letter h indicates four coordinately expressed polypeptides which are identical in size and differ only slightly in charge. Letter v indicates two only partially separated polypeptides.

Fig. 5 shows the time courses for representatives of the first group, which consists of newly appearing polypeptides that were synthesized early after elicitor treatment and became detectable within 2 h. The time courses for the second group of polypeptides, which disappeared as rapidly as the first group appeared, are shown in Fig. 6. All of these early changes in protein synthesis were more or less transient.

Comparatively late changes in the pattern of protein synthesis were also observed. A third group comprised about 14 polypeptides which were newly synthesized with high rates at late time points (Fig. 3C, arrows). The time courses for two representatives of this group are shown in Fig. 7. A fourth group consisted of polypeptides (Fig. 3C, circles) whose rates of synthesis decreased at similar, late time points (data not shown).

The fifth group is formed by many polypeptides which were not appreciably affected by the elicitor treatment. These polypeptides served as reference points throughout the analysis. Untreated control cells did not show any detectable change in protein synthesis within 30 h, thus excluding ageing of the cell culture as a possible cause of the observed late changes.

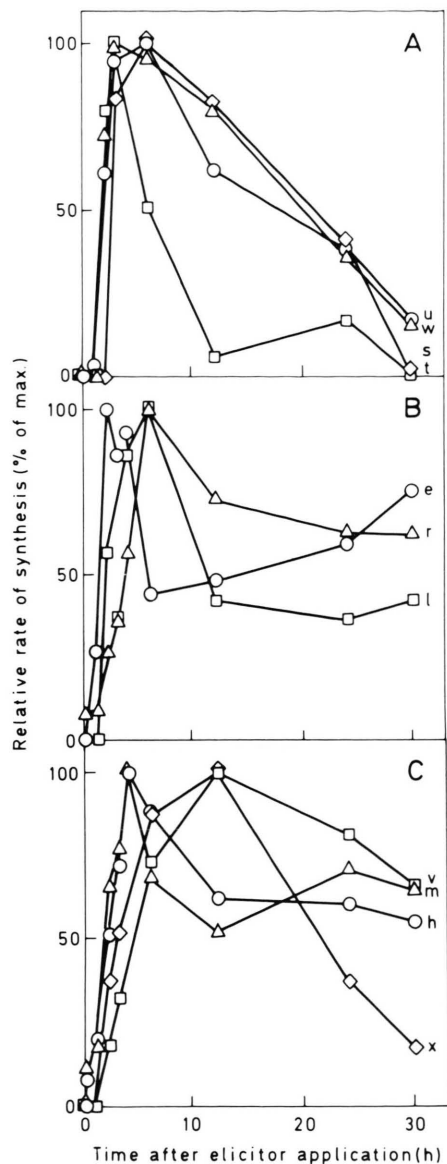


Fig. 5. Timing of changes in synthesis rates for early induced proteins (see Fig. 4 for identification). Proteins were *in vivo* pulse-labelled with [35 S]methionine 1 h prior to harvest at the indicated times and separated on two-dimensional gels. The relative rates of synthesis of each individual protein at each time point were determined on the resulting fluorographs by computer scanning and are plotted *versus* time. Due to saturation of the fluorographic response, maximal rates of synthesis may have been underestimated to some extent for heavily labelled polypeptides (e.g. e, h, m, r and v).

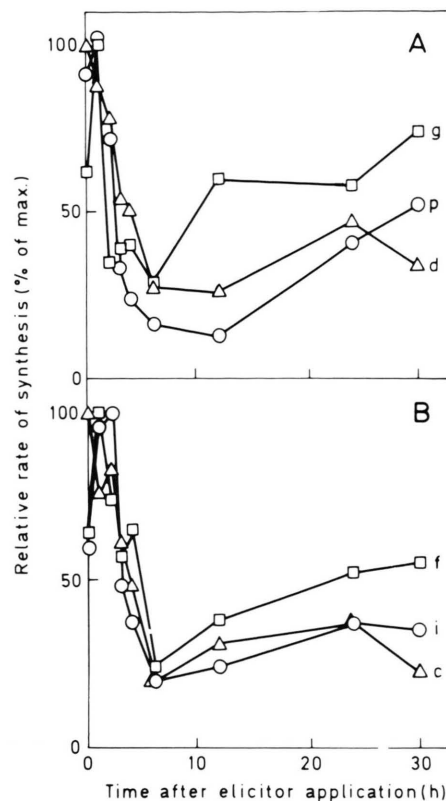


Fig. 6. Timing of changes in synthesis rates for early repressed proteins. See Fig. 4 and 5 for details.

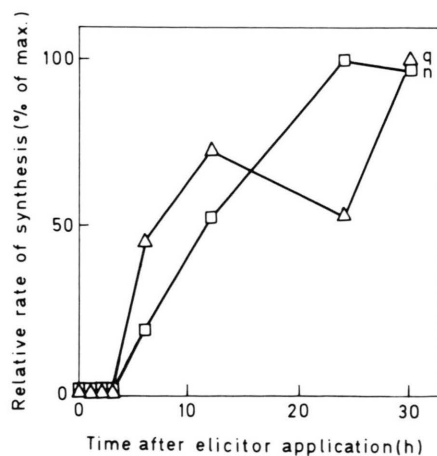


Fig. 7. Timing of changes in synthesis rates for late induced proteins. See Fig. 4 and 5 for details.

Identified representatives of early induced polypeptides

Previously identified members of the group of early induced polypeptides in elicitor-treated parsley cells are various isoforms of PAL [13] and 4CL [22]. Our present results indicate that PR1 and PR2, whose migration patterns on two-dimensional gels are indicated in Fig. 1 and Fig. 3, show a transient induction behaviour similar to that of PAL and 4CL. The time courses of PR1 and PR2 synthesis are included in Fig. 5 (polypeptides u and t). Like several other PR proteins of parsley (polypeptides s, v, w and x in Fig. 4), PR1 and PR2 clearly belong to the group of early synthesized polypeptides.

Identified representatives of late induced polypeptides

We have previously shown that the induction by elicitor of the enzymatic activity of BMT, catalyzing two terminal methylation steps of coumarin biosynthesis, is delayed when compared to, for example, PAL and 4CL [1]. We investigated whether this reflects a delayed synthesis of BMT protein. Fig. 8 shows the identification of two BMT polypeptides and the timing of their synthesis. Using a combination of two-dimensional gel electrophoresis and immunoblotting, two polypeptides, identical in size ($M_r = 42,000$) but with different isoelectric points of 6.4 and 6.7, stained specifically with antiserum against BMT (Fig. 8A).

As the separated polypeptides were radioactively labelled, autoradiography of the nitrocellulose filter revealed their relative position with respect to the rest of the polypeptides synthesized (Fig. 8B). This in turn allowed to identify BMT on a time-series of two-dimensional separations of *in vivo* pulse-labelled polypeptides (arrows in Fig. 8C), and to determine the time courses of their synthesis. Synthesis of the more acidic polypeptide started to increase 12 h after elicitor treatment and reached its maximal rate at late time points, showing it to be a member of the group of late induced polypeptides. The unambiguous attribution of the more basic polypeptide to either the early or the late group is not possible due to the presence of overlapping proteins that interfere with the analysis.

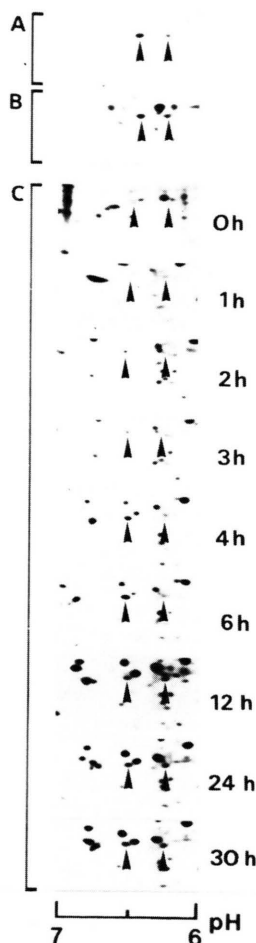


Fig. 8. Immunological identification and time course of synthesis of BMT. A, immunoblot with BMT antiserum using labelled proteins that had been extracted 30 h after elicitor application; B, autoradiography of A; C, time course of BMT synthesis. Polypeptides were *in vivo* pulse-labelled at the indicated times, as detailed in Fig. 5. Arrowheads point to the location of BMT polypeptides. Relevant sections of two-dimensional gels are shown (M_r approx. = 35,000–50,000).

Changes in total protein composition

Silver staining of proteins extracted 30 h after elicitor application showed, apparently as a consequence of the changes in the protein synthetic activity of the cells, a considerable alteration in protein composition (Fig. 9). A substantial part of the protein population consisted of *de novo* synthesized polypeptides (Fig. 9B, arrows), whilst other

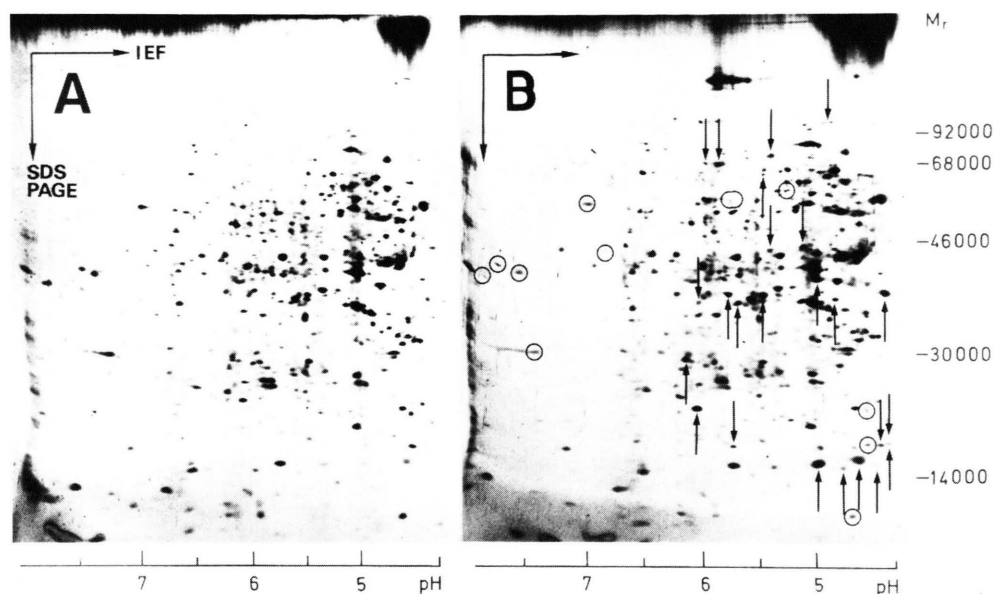


Fig. 9. Staining patterns of proteins with silver nitrate. A, untreated control; B, 30 h after elicitor application. Arrows indicate increased, circles indicate decreased abundance in elicitor-treated cells.

polypeptides, present in considerable amount in control cells (Fig. 9 A), became minor constituents in elicitor-treated cells (Fig. 9 B, circles).

Discussion

We have shown that suspension-cultured parsley cells undergo substantial alterations in protein synthesis upon treatment with elicitor. The analytical tool was a series of two-dimensional polyacrylamide gels. The intensity of each spot on the resulting fluorographs was taken as a measure of the rate of expression of a specific gene encoding an extractable, neutral or acidic protein. The entirety of spots represents an image of the corresponding translatable mRNA activity and thus of the state of expression of these genes at a given time point. The selection of genes analyzed by this method is unbiased as all genes coding for proteins of this type are detected, provided they are expressed at a certain minimal rate at some time during the experiment.

By this analytical method, we have identified five types of response which differ by the timing of effects on protein synthesis. Two groups of poly-

peptides were newly synthesized after elicitor application. The synthesis of the first group of early induced proteins became apparent about 2 h after elicitor application. The induction was transient with a maximum rate of synthesis at about 6 h. Transcriptional control has been established previously for several representatives of this group, PAL and 4CL [10, 13] as well as PR 1 and PR 2 [14]. Our analysis extends the number of proteins belonging to this group to at least about 25.

Synthesis of the other group of about 10 induced proteins started 4–6 h after administration of elicitor. In contrast to the first group, their rates of labelling remained high for at least 24–30 h. The differences in timing between these two groups were very likely to represent differences in timing of the transcriptional activation of the respective genes. This view is supported by recent *in vitro* run-off transcription experiments, which indicated the existence of at least two classes of genes differing in the timing of their elicitor-induced activation [6].

In addition to increased rates of protein synthesis, we detected two groups of proteins with reduced rates of synthesis. A similar type of dual re-

sponse has been described for proteins from elicitor-treated bean cells [23]. We show here, at least for parsley cells, that repression is as transient as induction. It appears that the timing of repression is inversely correlated with the timing of induction.

It seems most likely that the reduction of protein synthesis is also transcriptionally regulated. Alternative explanations, such as an elicitor-induced change in mRNA stability, similar to the iron-dependent regulation of ferritin and transferrin receptor synthesis in mammals [24], cannot be ruled out with certainty. However, repression at the transcriptional level of the small subunit of ribulose 1,5-bisphosphate carboxylase in potato leaves, infected with *Phytophthora infestans* or treated with an elicitor preparation from this fungus, has recently been demonstrated [25]. Rapidly decreased synthesis of this and a number of other transiently dispensable proteins may liberate protein synthetic capacity required to fully establish the defense response of elicitor-treated or pathogen-infected plant cells. The synthesis of the majority of proteins, constituting a final category, was not appreciably affected by elicitor, possibly indicating their role as indispensable housekeeping proteins.

At least the group of early induced proteins appears to be functionally heterogeneous. While PAL and 4CL are members of the general phenylpropanoid pathway, the functionally unidentified PR proteins cannot belong to that pathway. They are induced by elicitor but not by UV light, whereas the general phenylpropanoid pathway is activated by both stimuli. This, however, does not exclude the possibility that PR proteins, or other early induced proteins, are functionally related to the general phenylpropanoid pathway. Indirect evidence points to the early activation of a branch pathway that results in the accumulation of phenolics in cell walls around infection sites [3]. The only function attributable so far to a late induced protein is the previously established role of BMT

in the phenylpropanoid branch pathway that leads to furanocoumarin synthesis [1, 26].

Elicitor treatment of a plant cell culture allows molecular aspects of pathogen defense-related reactions to be studied without the experimental complications frequently encountered in analyzing a true plant-pathogen interaction. It is critical, however, that the cell culture maintains essential features of the plant-pathogen interaction. All reactions analyzed so far in elicitor-treated parsley cell suspension cultures were strikingly similar to those observed with the live fungus [4, 5, 27]. This may suggest that the overall temporal patterns of differential gene activation and inactivation, exemplified in their large complexity in this study, indicate the occurrence of similar, sequential reactions in the multicomponent disease resistance response of intact parsley plants.

However, additional spatial and tissue-specific components contribute to the complexity of the defense response of intact plant tissue. In contrast to suspension cultures, where essentially all cells are much more synchronously and homogeneously exposed to elicitor, attempted fungal penetration is highly localized. Extensive histochemical studies [3–5, 27] established elaborate spatial patterns of gene expression around sites of attempted infection in parsley leaves. In addition, leaf cells in direct contact with the fungus respond with locally restricted, hypersensitive death. This drastic response is apparently not exhibited by suspension-cultured cells under the conditions used here. It is therefore the more surprising how closely the response of elicitor-treated cell cultures mimics the response of pathogen-infected leaf tissue. Thus, our present results may be regarded as one further step towards an understanding of the complex plant-pathogen interactions.

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