Rapid Response of Suspension-cultured Parsley Cells to the Elicitor from *Phytophthora megasperma* var. *sojae*¹

INDUCTION OF THE ENZYMES OF GENERAL PHENYLPROPANOID METABOLISM

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**ABSTRACT**

Large and rapid increases in the activities of two enzymes of general phenylpropanoid metabolism, phenylalanine ammonia-lyase and 4-coumarate-CoA ligase, occurred in suspension-cultured parsley cells (*Petroselinum hortense*) treated with an elicitor preparation from *Phytophthora megasperma* var. *sojae*. Highest enzyme activities were obtained with an elicitor concentration similar to that required for maximal phenylalanine ammonia-lyase induction in cell suspension cultures of soybean, a natural host of the fungal pathogen.

The changes in phenylalanine ammonia-lyase activity in parsley cells were caused by corresponding changes in the mRNA activity for this enzyme. Phenylalanine ammonia-lyase mRNA activity increased much faster and transiently reached a much higher level in elicitor-treated than in irradiated cell cultures. In contrast to irradiation, treatment of the cells with the elicitor did not induce the enzymes of the flavonoid glycoside pathway, as demonstrated for acetyl-CoA carboxylase and chalcone synthase. Induction of these enzymes by light was abolished by simultaneous application of the elicitor.

Irradiation with UV light (13) and dilution into fresh medium (15) or water (14) induce the enzymes of general phenylpropanoid metabolism (group I) in cell suspension cultures of parsley. A second group of enzymes (group II), which converts the major product of the group I enzymes, 4-coumaroyl-CoA, to flavonoid glycosides, is induced with group I under the conditions of irradiation (8, 13), but not after dilution of a culture in the absence of light (14,15). Each group of enzymes is induced in a coordinated manner, irrespective of the mode of induction (8, 13–15, 17). One seemingly invariable characteristic of the induction was the occurrence of an apparent lag period which preceded the increases in enzyme activity (8, 12, 13). In irradiated or diluted cultures, a lag of about 2 to 2.5 h has always been observed for the enzymes of group I.

We have now treated parsley cell cultures with a crude elicitor preparation from *Phytophthora megasperma* var. *sojae* (Pms²) as a third method to induce the enzymes of group I. A very efficient induction of PAL by microbial interaction with cultured parsley cells was first discovered with a microorganism which was tentatively identified as a *Xanthomonas* species (30). More recently, Pms elicitor was shown to induce PAL in parsley cell cultures with similar efficiency as in cell cultures from a soybean cultivar, Glycine max cv. Harosoy 63, which is resistant to several races of the fungal pathogen (7). Resistance of seedlings from this plant to Pms has been related, at least in part, to the accumulation in infected tissue of isoflavonoid phytoalexins, such as glyceollin (7, 18). The initial reactions in the synthesis of these compounds are catalyzed by the enzymes of group I (6, 29).

Our interest in the interaction between the Pms elicitor and parsley cells arose especially from the observation that PAL induction was a very rapid response, when compared with the effects of irradiation or dilution. We report here that Pms mRNA activity increased almost immediately and very steeply upon treatment of the cells with elicitor. In addition, our present results provide another example of the close coordination which is involved in the induction of the group I enzymes in parsley cells.

**MATERIALS AND METHODS**

Cell Cultures. Cell suspension cultures of parsley (*Petroselinum hortense*) were propagated in medium I (11) containing 1 mm CaCl₂. Details of the procedure, including the conditions of irradiation with white light from fluorescent lamps, have been described (13). Cultures were used for experiments when the conductivity of the medium had reached a value between 1.5 and 2.0 mmho (12).

Preparation of Elicitor. “Large extracellular elicitor” was isolated after growth of Pms on liquid medium as described (2). Stock solutions of the non-dialyzable material were autoclaved and used as elicitor preparations. About 20% of this material was anthrone-positive carbohydrate, as determined by the Dische reaction (5).

Enzyme Assays. Standard procedures were used for preparing crude cell extracts (13) and measuring the activities of PAL (31), 4CL (20), ACC (8, 16), and CHS (26). The data are expressed in µkat/kg of protein. One unit of enzyme activity (1 kat) is the amount of enzyme required for the formation of 1 mol of product in 1 s under the assay conditions.

**Determination of Rates of Enzyme Synthesis.** The methods for for

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³ Abbreviations: Pms, *Phytophthora megasperma* var. *sojae*; PAL, phenylalanine ammonia-lyase (EC 4.3.1.5); 4CL, 4-coumarate-CoA ligase (EC 6.2.1.12); ACC, acetyl-CoA carboxylase (EC 6.4.1.2); CHS, chalcone synthase (previous name: flavanone synthase).
the determination of the rates of PAL and CHS synthesis \emph{in vivo} or of PAL synthesis \emph{in vitro} were the same as used previously (4, 25, 27).

\textbf{Cyanogen Bromide Cleavage Products.} Radioactive PAL was prepared by adding 100 \(\mu\text{Ci}\) of a mixture of 15 \(^{14}\text{C}\)-labeled L-amino acids (New England Nuclear) to 20-ml samples from a cell culture. One sample was continuously irradiated for 10 h, the other treated with Pms elicitor (30 \(\mu\text{g}\)/ml) for 8 h, and the cells were then harvested. The radioactivity was added to each sample in four equal portions at 1-h intervals between 5 and 2 h prior to harvest. The enzyme was immunoprecipitated, purified by disc-gel electrophoresis, eluted from the gel and co-precipitated with BSA which was added to a final concentration of 0.5 mg/ml (3). Cyanogen bromide cleavage (10) in 70% formic acid was performed separately with 25,000 cpm of each of the two PAL preparations. The products were analyzed on 20% polyacrylamide slab gels (22) by autoradiography for 3 weeks with Kodak K-0-Mat film.

\textbf{Protein Determination.} Protein was determined either by the Biuret method (21) or by the method of Schaffner and Weissmann (23).

\textbf{Determination of Radioactivity.} Radioactivity was measured by scintillation counting in toluene containing 5 g of 2,5-diphenyloxazole (PPO)/l or in a mixture of toluene and Triton X-100 (2:1, \(v/v\)) containing 5 g of PPO/l.

\section*{RESULTS}

\textbf{Dependence of Enzyme Induction on Elicitor Concentration.} The extent to which PAL and 4CL were induced during an 8-h incubation of parsley cell cultures with the Pms elicitor was dependent on the amount of elicitor added (Fig. 1). Maximal induction of both enzymes was obtained with 30 \(\mu\text{g}\)/ml of the elicitor. The effect was only slightly smaller with 10 \(\mu\text{g}\)/ml. This elicitor concentration was used throughout the following experiments if not stated otherwise.

We have frequently noted that absolute values for induced enzyme activities could be directly compared only when the same batch of cells was used. This phenomenon has previously been observed for induction by irradiation or dilution, and has now also been found for incubations with the elicitor.

The control value for PAL activity shown in Figure 1 is high compared with previous results and is attributed to the mode of manipulation of cell cultures in these experiments (see "Discussion"). In contrast to PAL, 4CL has a relatively high base level of activity even in completely untreated cultures (13). The reason for this phenomenon has not been examined. However, we assume that PAL and 4CL activities below the broken line in Figure 1 were not induced by the elicitor, whatever the cause of the high control levels might be. To facilitate a comparison of the results, enzyme activities obtained with control cells were subtracted in all of the following experiments from activities obtained after specific treatments. On average, the activities in extracts from completely untreated cells were 0–3 \(\mu\text{kat}\)/kg for PAL and 25–35 \(\mu\text{kat}\)/kg for 4CL; ACC and CHS activities were undetectable with the assays used.

\textbf{Kinetics of Enzyme Induction.} The changes in PAL and 4CL activities in response to treatment of parsley cells with Pms elicitor are shown in Figure 2A. The same relative changes were observed for both enzymes. A comparison of the resulting curve with that obtained for light-induced changes in PAL and 4CL activities shows that the response to elicitor was much more rapid than the response to light (Fig. 2B). Half-maximal PAL and 4CL activities (corrected for noninduced levels) were reached about 3 h after

\begin{figure} [h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Levels of PAL (\(\bigcirc\)) and 4CL (\(\triangle\)) activities obtained within 10 h of treatment of suspension-cultured parsley cells with various concentrations of Pms elicitor. The broken line indicates the level of enzyme activity in the control cultures which is, in part, due to enzyme induction by using fresh glass vessels for the incubations (see text for further explanations).}
\end{figure}

\begin{figure} [h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Changes in PAL (\(\bigcirc\)) and 4CL (\(\triangle\)) activities following treatment of parsley cells with Pms elicitor (A) or light (B). All values were corrected for levels of enzyme activity in untreated cells (\(\leq 3 \mu\text{kat}\)/kg for PAL and 28 \(\mu\text{kat}\)/kg for 4CL). The dotted line in B represents the same curve as shown in A. Note the change in scale for 4CL activity from A to B by a factor of about 2/3.}
\end{figure}
addition of the elicitor, a time when the light effect was hardly detectable. A peak for both enzyme activities was observed several hours earlier with elicitor-treated cells than with irradiated cells.

The induction was further studied at the mRNA level, using PAL as an example. A sensitive assay for PAL mRNA activity was originally established for the light-induced enzyme (24, 27). The antisera used for this assay was now found to precipitate elicitor-induced PAL and light-induced PAL with similar efficiency. The immunoprecipitated radioactive enzymes were indistinguishable on polyacrylamide gels under denaturing conditions. A comparison of the cyanogen bromide cleavage products from the two immunoprecipitated PAL species showed similar patterns for the distribution of radioactivity on a polyacrylamide gel. This suggested that both treatments led to the induction of similar, or perhaps identical, enzyme proteins.

The activity of PAL mRNA in a reticulocyte lysate incubated with polyribosomal extracts from elicitor-treated cells was very high at 3 h after the onset of induction (51,300 cpm/mg RNA) and decreased to about one-tenth of this activity at 10 h. In contrast, light-induced PAL mRNA activity increased from the 3rd h (4,500 cpm/mg RNA) to the 10th h (12,000 cpm/mg RNA) in the usual manner (24, 25, 27). Thus, the total amount of PAL mRNA activity extractable from elicitor-treated cells at 3 h was about four times that extractable from cells which had been continuously irradiated for 10 h. In this particular experiment, PAL activity at 10 h was higher for elicitor-treated cells (93 µkat/kg) than for irradiated cells (61 µkat/kg).

As in previous studies (4, 25, 27), the following more detailed measurements of PAL mRNA activity were conducted in vivo, because more accurate data could be obtained than in vitro. Figure 3A shows the changes in the rate of PAL synthesis in vivo during a period of 13 h following treatment of parsley cells with Pms elicitor. Portions from the same cell culture as used above (Fig. 2) were labeled for 30 min with [35S]methionine at the times indicated, and radioactive PAL mRNA was immunoprecipitated and analyzed by SDS gel electrophoresis. Again, an analogous experiment with irradiated cells was included for comparison (Fig. 3B) and gave results very similar to those obtained previously (4, 27). The shapes of the two curves for PAL synthesis in elicitor-treated and irradiated cells differed markedly. In particular, the rate of synthesis increased very rapidly during the first 2 h after elicitor treatment, when the increase was comparatively slow in irradiated cells, and a relatively early peak in elicitor-treated cells was followed by a sharp decline.

The corresponding, large difference between the changes in PAL activity under the two conditions of induction is illustrated by the broken curves in Figure 3. The data represented by these curves were obtained by calculating the expected changes in enzyme activity (E) from the observed changes in the rate of synthesis (dE/dt), using the equation

\[ \frac{dE(t)}{dt} = k_e(t) - k_d E(t), \]

as described elsewhere (4, 27). The rate of PAL degradation (k_d) was calculated from the previously determined half-life of about 7 h (27). Within experimental error, the calculated curves coincided with the experimentally determined data (taken from Fig. 2), both in elicitor-treated and irradiated cells.

Comparison of Group I and Group II Enzymes. In another experiment (Table I), we have compared the effects of elicitor, light, or both, given simultaneously, on PAL and on the first two enzymes of group II, ACC and CHS. The patterns of enzyme induction after 3 h and 10 h of treatment indicate that the two effects are not only less than additive, but rather seem to impair each other.

For PAL, the effects observed either with elicitor or with light alone were similar to those shown above. As in Figure 2, similar levels of PAL activity were obtained after 10 h of either treatment. When both treatments were combined, the early induction of PAL observed at 3 h with elicitor-treated cells was not significantly affected by simultaneous application of light, which alone had no detectable effect. At 10 h, however, the combined treatments resulted in a considerable reduction of the effect observed with elicitor or light alone. Essentially the same result was obtained in a second experiment with a different batch of cells.

Treatment of the cells with elicitor alone had no measurable effect on the enzymes of group II (Table I). Induction of ACC and CHS by light was normal (8,13), i.e. a large increase was found 10 h after the onset of irradiation. However, simultaneous application of elicitor and light caused a drastic inhibition of the light effect.

The differential effects of elicitor and light on the enzymes of group II were further substantiated by the results shown in Figure
Table 1. Effects of Various Treatments of Cultured Parsley Cells on Enzymes of Group I (PAL) and Group II (ACC, CHS)
Maximal activities (corrected for noninduced levels) were 73 µkat/kg for PAL, 9.5 µkat/kg for ACC, and 1.6 µkat/kg for CHS.

<table>
<thead>
<tr>
<th>Time After Onset of Induction</th>
<th>Treatment</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PAL</td>
</tr>
<tr>
<td>h</td>
<td></td>
<td>% of maximum</td>
</tr>
<tr>
<td>0</td>
<td>None (control)*</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Elicitor</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Elicitor + light</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>Elicitor</td>
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</tr>
<tr>
<td></td>
<td>Light</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Elicitor + light</td>
<td>60</td>
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</tbody>
</table>

*Enzyme activities in untreated cells were subtracted from all values.

Fig. 4. Rates of CHS synthesis in vivo in parsley cells treated either with light (□) or with Pms elicitor (■). The same [%S]methionine-labeled cells were used as for the experiment shown in Figure 3. The procedure for measuring the rates of labeling of the enzyme subunit with a CHS-specific antiserum (27) was essentially the same as that described for PAL subunits.

4. CHS, the key enzyme of group II (26), was not synthesized to a detectable degree in elicitor-treated cells, while in irradiated cells from the same culture the rate of CHS synthesis increased greatly between 2 and 13 h, a result which is in close agreement with previous observations (27).

Phenylpropanoid Compounds in Elicitor-Treated Cells. The absence of group II enzyme activity in parsley cells treated with Pms elicitor suggested that phenylpropanoid derivatives other than flavonoids were formed as a result of increased group I enzyme activity. Several as yet unidentified UV-absorbing compounds were observed on chromatograms after acid hydrolysis, followed by extraction with ethyl acetate, of both ethanolic extracts and the ethanol-insoluble residues of cells. However, all of the clearly visible spots were present on the chromatograms, regardless of whether or not the cells had been treated with light or elicitor for periods of up to 20 h. None of these spots co-migrated with either unsubstituted, 4-hydroxy-, 3,4-dihydroxy-, or 3-methoxy-4-hydroxy-substituted benzoic or cinnamic acids. No prominent spot was found under these conditions which would have occurred specifically in elicitor-treated cells.

The low base level of the two reductases of the lignin pathway (9) remained unaffected upon treatment of the cells with Pms elicitor, and a phloroglucinol-HCl test (28) was negative in both treated and untreated cells.

A striking change in color was observed with elicitor-treated cells. Within 10 to 30 h, the cell cultures turned first yellow and then brownish-yellow. Untreated cultures were almost colorless. The brownish-yellow color was associated with only a small percentage of the elicitor-treated cells. Colored cells occurred in a seemingly random fashion either as single cells or as members of aggregates ranging widely in size from two to several hundreds of cells. All colored cells appeared dead, and the color was associated with the structurally disintegrated interior of the cells, as judged from a microscopic examination of a cell culture after 30 h of treatment with the elicitor.

DISCUSSION

Our present results provide another example of the broad specificity of an elicitor from a plant pathogen (1). Although parsley is not a natural host for Pms, the Pms elicitor induces the two group I enzymes, PAL and 4CL, very efficiently in cultured parsley cells. Similar concentrations of the elicitor were required for maximal enzyme induction in cell cultures from parsley (Fig. 1) and from soybean, the natural host of the pathogen (7).

We have shown that the increase in PAL activity upon elicitor treatment is due to an almost immediate increase in the rate of PAL synthesis (PAL mRNA activity). The results of mathematical calculations presented here (Fig. 3) and in earlier reports (4, 25, 27) suggest that large and rapid changes in mRNA activity and an essentially constant rate of enzyme degradation regulate PAL activity in parsley cells under all known conditions of induction, i.e. by irradiation, dilution or treatment with Pms elicitor. It is important to note that small changes in the rate of PAL degradation, e.g., by a factor of two (4), would not alter this conclusion. The data further demonstrate that, under the conditions used, PAL mRNA induction is much more efficient with Pms elicitor than with light. About a 2- to 4-fold higher specific activity for PAL mRNA was obtained with the elicitor in several independent experiments.

A high control value for PAL activity in cells which had not been treated with the elicitor was obtained in the experiment shown in Figure 1. Such high values in control cells have always been observed in experiments where all cultures, including controls not receiving elicitor or light, were subjected to the same procedure of incubating cells in small portions for several hours in fresh glass vessels. This effect has been attributed to the adsorption to the glass wall of one or more compounds which occur in both the cells and the culture medium and are involved in the regulation of PAL synthesis (B. Betz and K. Hahlbrock, unpublished results). A similar mechanism has been postulated to explain the effect of dilution (14).

One remarkable difference between PAL and 4CL is the amount of activity present in completely untreated cell cultures. The noninduced level of PAL has always been slightly above the detection limit (12) and can be maximally stimulated about 100-fold. In contrast, the specific activity of 4CL in crude extracts from untreated cells varies from culture to culture and is usually at least about 10 times higher than that of PAL. Maximal induction of 4CL above this relatively high base level ranges from 3- to 10-fold, with the highest specific activity in vitro reaching in some experiments up to twice the amount obtained for PAL (e.g., Fig. 2A).

The highest absolute amounts of 4CL activity, relative to PAL activity, which could be obtained either with light or with the
elicitor differed markedly. In one of several related experiments performed in this study (Fig. 2), the elicitor was almost twice as efficient in the induction of 4CL as was light, whereas both treatments resulted in similar amounts of PAL activity.

Another unexpected result was the apparent impairment of the light effect by the elicitor. A particularly remarkable observation was the almost complete abolition of the induction by light of the group II enzymes in the presence of the elicitor. The data for PAL (Table 1) indicate that the two effects also impair each other with regard to the group I enzymes. The reason for this phenomenon is unknown.

The most surprising observation was the very rapid response of parsley cells to the Pms elicitor in the induction of PAL and 4CL, as compared with the induction by irradiation or dilution of the cultures. The pronounced lag period preceding detectable increases in both enzyme activities in irradiated or diluted cell cultures was almost completely abolished when the cells were treated with the elicitor. It appears that the induction of general phenylpropanoid metabolism is a direct response of parsley cells to the elicitor and not a slow secondary effect. Moreover, the high degree of coordination in the induction of the group I enzymes which has previously been observed with irradiated or diluted parsley cell cultures has now also been demonstrated for PAL and 4CL induction by Pms elicitor, despite the different kinetics of induction. Evidence for a coordinated induction of PAL and 4CL by the elicitor has been obtained at two different levels. First, the degree of induction of both enzymes showed the same dependence on the concentration of elicitor in the culture medium; and second, the time course of induction was the same for PAL and 4CL.

Concerning the coordinated induction of PAL and 4CL in differently treated parsley cells, we draw the following conclusions from a combination of the present and previous results.

(a) The timing of increases in the enzyme activities is always coordinated, irrespective of the length of an apparent lag period, and regardless of whether irradiation, dilution, or treatment of the cell cultures with elicitor is the mode of induction.

(b) The high degree of coordination in the induction of PAL and 4CL is also concluded from similar variations in the extent of their induction either with the growth stage of the culture, when light is the inducing agent (17), or with the elicitor concentration in a given batch of cells (Fig. 1).

(c) The coordination in the induction of PAL and 4CL might be less pronounced with respect to the absolute amounts of catalytic activity formed within a given period of time. However, the measurement of different maximal amounts of 4CL activity, relative to PAL activity, in irradiated and elicitor-treated cell cultures does not necessarily result from different relative rates of PAL and 4CL synthesis following the two treatments. An alternative possibility would be a difference in the rates of degradation of the two enzymes. Furthermore, the occurrence of 4CL isoenzymes which are induced differentially upon different treatments of the cells must be considered. Isoenzymes of 4CL have been demonstrated in various systems, including cultured soybean cells (19), but so far not in parsley cells (20). The specific involvement of 4CL isoenzymes in different phenylpropanoid pathways, such as lignin, cinnamate ester, or flavonoid biosynthesis, has been postulated (20).

(d) Induction of the group I enzymes can occur without the simultaneous induction of the group II enzymes or the enzymes of the lignin pathway. This has previously been shown for diluted cultures (15) and has now been confirmed with elicitor-treated parsley cells. This result leaves open the question as to the function of the group I enzymes upon elicitor treatment if neither flavonoids nor lignin are formed. Cell cultures from soybean, the natural host of Pms, respond to the elicitor by the accumulation of flavonoid phytoalexins, e.g. glyceollin (7), and the induction of both group I and group II enzymes prior to the production of these compounds in elicitor-treated soybean cotyledons has been demonstrated (6, 29). Our present results might suggest that the group I enzymes play an additional role, besides flavonoid or lignin production, in plant-parasite interactions.

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