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Induction of Defense Responses in Cultured Parsley Cells by Plant Cell Wall Fragments[1]

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ABSTRACT

Cell suspension cultures of parsley (*Petroselinum crispum*) accumulated coumarin phytoalexins and exhibited increased β-1,3-glucanase activity when treated with either a purified α-1,4-D-endopolygalacturonic acid lyase from *Erwinia carotovora* or oligogalacturonides solubilized from parsley cell walls by endopolygalacturonic acid lyase. Coumarin accumulation induced by the plant cell wall elicitor was preceded by increases in the activities of phenylalanine ammonia lyase (PAL), 4-coumarate:CoA ligase (4CL) and *S*-adenosyl-L-methionine:xanthotoxol *O*-methyltransferase (XMT). The time courses for the changes in these three enzyme activities were similar to those observed in cell cultures treated with a fungal glucan elicitor. The plant cell wall elicitor was found to act synergistically with the fungal glucan elicitor in the induction of coumarin phytoalexins. As much as a 10-fold stimulation in coumarin accumulation above the calculated additive response was observed in cell cultures treated with combinations of plant and fungal elicitors. The synergistic effect was also observed for the induction of PAL, 4CL, and XMT activities. These results demonstrate that plant cell wall elicitors induce at least two distinct biochemical responses in parsley cells and further support the role of oligogalacturonides as important regulators of plant defense.

A number of studies have been published that support the hypothesis that oligosaccharides solubilized from plant cell walls are physiologically important regulators of plant defense responses (2, 16). Oligogalacturonides that induce phytoalexin production in soybean cotyledons can be solubilized from soybean cell walls by partial acid hydrolysis (14) or by treatment with a purified PGA lyase[3] isolated from the phytopathogenic bacterium, *Erwinia carotovora* (3, 5). Similarly, oligogalacturonides obtained from citrus polygalacturonic acid by degradation with a fungal endopolygalacturonase induce casbene synthetase in castor bean (9). Other studies have indicated that oligogalacturonides induce the accumulation of phytoalexins in carrot cell cultures (12) and proteinase inhibitor I in tomato leaves (1, 16), as well as lignification in cucumber (15).

Recently, it was shown that a decagalacturonide that is an elicitor of phytoalexins in soybean cotyledons acts synergistically with another phytoalexin elicitor, a branched β-glucan obtained from fungal cell walls (6). These results suggest that oligogalacturonides may play an important role in modulating the induction of plant defense responses by enhancing the induction of defense responses by other elicitors.

To test whether the synergistic effect occurs in other systems as well, we have examined a few selected responses of suspension-cultured parsley cells (*Petroselinum crispum*) to treatment with oligogalacturonide preparations isolated from parsley cell walls. We chose this system because cultured parsley cells have been used extensively to study the induction of defense responses by fungal elicitors (7, 11). Several defense responses are activated in parsley cells treated with a fungal glucan elicitor, including the production of coumarin phytoalexins and the hydrolases, β-1,3-glucanase and chitinase (10). The accumulation of coumarin phytoalexins is correlated with the induction of enzymes involved in general phenylpropanoid metabolism, *e.g.* PAL and 4CL, and in the furanocoumarin biosynthetic pathway, *e.g.* XMT (8, 10, 11).

In this report, we demonstrate that PGA lyase, or oligogalacturonide preparations solubilized from parsley cell walls by PGA lyase, induce similar defense responses in cultured parsley cells. We also demonstrate that these oligogalacturonide preparations act synergistically with a crude fungal glucan elicitor in the induction of coumarin phytoalexins.

MATERIALS AND METHODS

Cell Cultures. Suspension-cultured parsley cells (*Petroselinum crispum*) were maintained as described (10). Experiments were performed with 400-ml cultures 7 to 8 d after transfer, when the conductivity of the medium was 2 to 3 mmho.

Elicitor Preparation. PGA lyase I was purified to apparent homogeneity as described (3) from culture filtrates of *Erwinia carotovora* f. sp. *carotovora* (ATCC No. 495). Enzyme activity was measured spectrophotometrically (3). One unit of enzyme activity is defined as the amount of enzyme needed to produce 1 μmol unsaturated GalA per min at 30°C.

A crude glucan elicitor obtained by autoclaving cell walls of the fungus, *Phytophthora megasperma* f.sp. *glycinea*, was prepared as described (10) and kindly provided by Erich Kombrink (Köln, FRG). This elicitor preparation is referred to in this report as fungal elicitor.

For the isolation of plant cell wall elicitors, parsley cell walls were prepared from 400-ml cultures 8 d after transfer as described (17) except that the walls were not treated with α-amylase. Cell walls (12.5 g) were obtained from 400 g fresh weight of parsley

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[3] Abbreviations: PGA lyase, α-1,4-D-endopolygalacturonic acid lyase (EC 4.2.2.2); PAL, phenylalanine ammonia lyase (EC 4.3.1.5); 4CL, 4-coumarate:CoA ligase (EC 6.2.1.12); XMT, *S*-adenosyl-L-methionine:xanthotoxol *O*-methyltransferase; GalA, galacturonic acid.

cells. Elicitor preparations were obtained by suspending 200-mg portions of cell walls in 20 ml of buffer (5 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.5) in sterile centrifuge tubes and adding 0.5 units of PGA lyase to each tube. The suspensions were incubated at 30°C and mixed every 30 min. At specified time intervals, the suspensions were combined and filtered through 2 layers of Whatman GF/C glass fiber discs. The filtrate was immediately titrated to pH 5.5 with 2 M HCl and the PGA lyase inactivated by heating at 100°C for 30 min. A_{235} measurements were made to estimate relative amounts of cell wall degradation by PGA lyase. The heat-treated filtrate was concentrated to about 10 ml by evaporation under reduced pressure after addition of 20 ml 1 M imidazole-HCl (pH 7.0). The concentrated preparation was filtered through 2 layers of Whatman No. 1 to remove precipitated material and desalted by gel permeation chromatography on a Sephadex G-10 column (2.5 × 70 cm) equilibrated with deionized H_2O. The desalted preparations contained oligosaccharides with degrees of polymerization of three or greater.

The experiments presented were performed with two different cell wall elicitor preparations, which are referred to as plant elicitors I and II. Plant elicitor I was obtained from cell walls treated with PGA lyase for 2.5 h (A_{235} = 0.735, 72 mg GalA eq obtained from 3 g cell walls) while plant elicitor II was obtained from an identical 3.0-h digest (A_{235} = 1.099, 114 mg GalA eq obtained from 2.4 g cell walls). Experiments were repeated at least twice; the data presented are from representative experiments unless otherwise indicated.

Elicitor Treatment of Cell Cultures. Twenty-ml aliquots of cell culture were aseptically transferred to autoclaved 125 ml Erlenmeyer flasks containing fungal and/or plant elicitors dissolved in 5 ml H_2O. In experiments with PGA lyase, the enzyme solution was filter-sterilized prior to addition to the autoclaved flasks. Cells were incubated at 25°C in the dark on a shaker operating at 100 rpm. Cells were collected by suction filtration on two layers of Whatman No. 1 filter paper, weighed, frozen in liquid N_2, and stored at −20 or −70°C. Culture medium was extracted immediately or stored at −20°C.

Extraction and Analysis of Coumarins in Culture Medium. Culture medium (15–20 ml) was extracted twice with 20 ml of $CHCl_3$. The extracts were combined and evaporated to dryness. The residue was dissolved in 1.0 ml spectrophotometric grade methanol and a 1:10 (v/v) dilution into methanol was prepared for A_{320} measurements. The data presented for coumarin accumulation in "Results" are the A_{320} measurements of the 1:10 dilutions. Aliquots of the undiluted methanol concentrates were analyzed by TLC on silica gel plates as described (10).

Extraction of Cells and Determination of Enzyme Activities. Cells were extracted for enzyme activity determinations as described (10). PAL and 4CL activities were determined with spectrophotometric assays (10). XMT activity was determined as described (8), except that the final concentration of *S*-adenosyl-L-methionine was 8.7 μM and the reaction volume was 100 μl. β-1,3-Glucanase activity was measured as described (10), except that the *p*-hydroxybenzoic acid hydrazide method (13) was used to quantitate reducing sugars.

Analytical Methods. Glycosyl composition, uronic acid content, and protein concentrations were determined as previously described (4).

RESULTS

Induction of Defense Responses by PGA Lyase. The response of parsley cells to treatment with PGA lyase was essentially the same as that observed in cells treated with fungal elicitor (10). The cells underwent extensive browning and exhibited decreased growth, as measured by fresh weight of the cells 48 h after treatment. Significant levels of coumarin phytoalexins accumulated in the medium within 48 h after treatment (Fig. 1). PGA lyase also induced β-1,3-glucanase activity in parsley cells. Extracts of cells treated with 22 munits/ml PGA lyase contained 2- to 3-fold higher levels of β-1,3-glucanase activity, relative to control cells treated with water. The ability of PGA lyase to induce coumarin accumulation and β-1,3-glucanase activity was abolished by boiling the enzyme preparation.

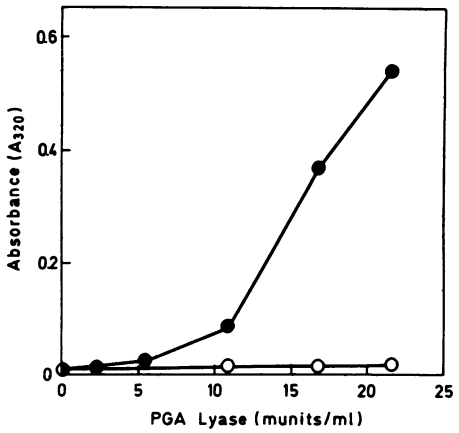

FIG. 1. Accumulation of coumarins in parsley cell cultures 48 h after treatment with different concentrations of untreated PGA lyase (●) or PGA lyase heated at 100°C for 30 min (○). Coumarins were extracted from the culture medium with $CHCl_3$ and quantitated by measuring the A_{320} of 1:10 dilutions of the concentrated extracts as described in "Materials and Methods." Medium extracts of a culture treated with 10 μg/ml fungal elicitor had an A_{320} of 2.042.

Release of Elicitors from Parsley Cell Walls by PGA Lyase. Initial experiments demonstrated that PGA lyase released elicitors of coumarin accumulation from parsley cell walls. Elicitor-active material was solubilized during the first several hours of incubation, reaching a maximum at 2 to 3 h. After 3 h of incubation, the elicitor activity decreased, suggesting that the elicitors were being degraded. This decrease in elicitor activity indicated that the elicitor-active molecules released from the cell walls contained α-1,4-linked GalA residues that were required for elicitor activity.

Two large-scale preparations of elicitor were obtained to further characterize the induction of defense responses by plant cell wall elicitors (plant elicitors I and II). These preparations contained greater than 90% GalA (w/w) and less than 1% (w/w) protein. Both of these preparations induced coumarin accumulation, with plant elicitor I having a 2-fold higher specific elicitor activity, based on GalA eq, than plant elicitor II (Fig. 2). TLC of culture medium extracts demonstrated that the coumarins induced by the plant elicitors were very similar to those induced by fungal elicitor (data not shown). Both plant elicitor preparations induced β-1,3-glucanase activities 3- to 4-fold above that measured in untreated cells. These two preparations also induced cell browning and decreased cell growth similar to that observed in cell cultures treated with fungal elicitor or PGA lyase.

Effects of Combinations of Plant Elicitor and Glucan Elicitor. Initial experiments indicated that both plant elicitors I and II acted synergistically with the fungal elicitor in the induction of coumarins in parsley cells. Since these experiments exhausted our supply of plant elicitor I, more detailed studies on the synergism were done with plant elicitor II. The synergistic activitation of coumarin induction by combinations of plant elicitor II and the fungal elicitor are presented in Figures 3 and 4. Combinations of 0.1 or 0.2 μg/ml fungal elicitor with 100 or 200 μg/ml plant elicitor II induced coumarins more efficiently than when these concentrations of elicitors were assayed separately. A 10-fold increase above the calculated additive response was observed when 0.1 μg/ml fungal elicitor was assayed in combination

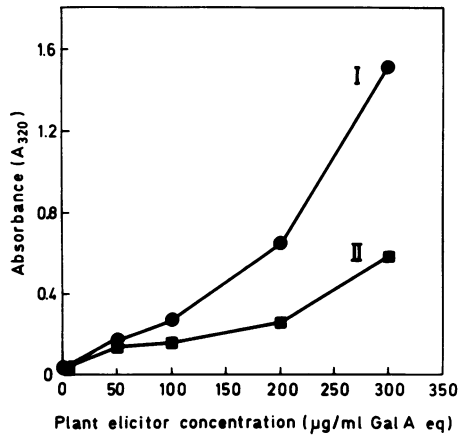

FIG. 2. Accumulation of coumarins in the medium of parsley cell cultures 48 h after treatment with different concentrations of plant elicitor I (●) or plant elicitor II (■). Coumarins were extracted from culture medium and quantitated as described in Figure 1. Medium extracts of a culture treated with 10 μg/ml fungal elicitor had an A_{320} of 2.168.

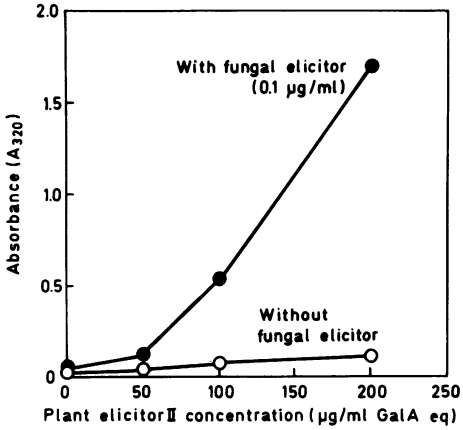

FIG. 3. Induction of coumarin accumulation in the medium of parsley cell cultures by the indicated concentrations of plant elicitor II assayed in the absence (○) or presence (●) of 0.1 μg/ml fungal elicitor. Coumarins were extracted from the culture medium and quantitated as described in Figure 1. Medium extracts of a culture treated with 10 μg/ml fungal elicitor had an A_{320} of 1.554.

with 200 μg/ml plant elicitor II (Fig. 3). Similarly, a 6-fold increase above the calculated additive response was observed when 0.1 μg/ml fungal elicitor was assayed in combination with 50 μg/ml plant elicitor I.

Coumarin and Enzyme Induction Kinetics. The induction kinetics of coumarins and selected enzymes involved in coumarin biosynthesis were measured to directly compare the responses of parsley cell cultures treated with plant and/or fungal elicitors. The time courses for the induction of coumarins, PAL, and XMT by plant elicitor II and the fungal elicitor are presented in Figure 5. Qualitatively, the induction kinetics for coumarins and enzyme activities were similar for both elicitors. PAL activity exhibited a rapid transient increase, reaching a maximum at approximately 12 h. 4CL activity increased transiently in parallel with PAL activity (data not shown). XMT activity in elicitor-treated cells increased steadily over the 48 h incubation period after an initial lag of approximately 4 h. The differences in the absolute levels of coumarin and enzyme activity induction by plant elicitor II and the fungal elicitor reflected the lower elicitor activity of 200 μg/ml plant elicitor II compared to 10 μg/ml fungal elicitor, which induced a maximum response (10).

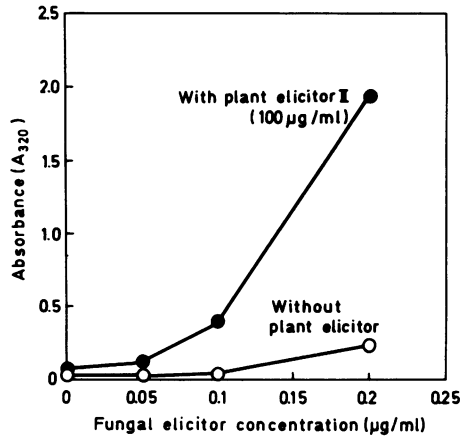

FIG. 4. Induction of coumarin accumulation in the medium of parsley cell cultures by the indicated concentrations of fungal elicitor assayed in the absence (○) of presence (●) of 100 μg/ml plant elicitor II. Coumarins were extracted from the culture medium and quantitated as described in Figure 1. Medium extracts of a culture treated with 10 μg/ml fungal elicitor had an A_{320} of 1.544.

The time courses for the induction of coumarins, PAL, and XMT in cells treated with a combination of 0.2 μg/ml fungal elicitor with 100 μg/ml plant elicitor II are presented in Figure 6. At these concentrations, neither elicitor alone induced significant coumarin accumulation (Fig. 6A) or XMT activity (Fig. 6C) and only small transient increases in PAL activity (Fig. 6B) were observed. In contrast, when assayed in combination, these concentrations of elicitors caused a rapid induction of PAL activity that was maintained for at least 48 h after treatment (Fig. 6B). Changes in 4CL activity were again similar to those observed for PAL. Coumarin accumulation and XMT activity were also strongly induced by the combination of elicitors, exhibiting an 8-fold induction over the calculated additive responses 48 h after treatment (Fig. 6, A and C). The time course for XMT induction by combinations of elicitors paralleled that of coumarin accumulation, and was similar to that observed in cells treated with higher concentrations of either elicitor alone (Fig. 5C).

DISCUSSION

We have demonstrated that oligosaccharides solubilized from parsley cell walls by the pectin-degrading enzyme, PGA lyase, induced at least two distinct defense responses in suspension-cultured parsley cells. Cultures treated with plant elicitor preparations accumulated coumarin phytoalexins identical to those produced by cells treated with fungal elicitor (8, 10), as well as β-1,3-glucanase, another putative defense response. As in cells treated with fungal elicitor, the accumulation of coumarin derivatives was associated with the induction of enzymes involved in their biosynthesis. Cell browning, which was also observed in cultures treated with plant elicitors, may be indicative of the additional activation of phenylpropanoid pathways other than coumarin synthesis (7).

The plant elicitor preparations contained predominantly GalA, suggesting that the elicitor-active components in these preparations were oligogalacturonides. This is consistent with the observation that prolonged incubation of cell walls with PGA lyase resulted in digests with reduced elicitor activity. Concentrations of 200 to 300 μg/ml plant elicitor were required to significantly induce defense responses in parsley cell cultures. These relatively high concentrations of plant elicitor were probably required because the elicitor-active components were likely to comprise only a small percentage of the oligogalacturonides

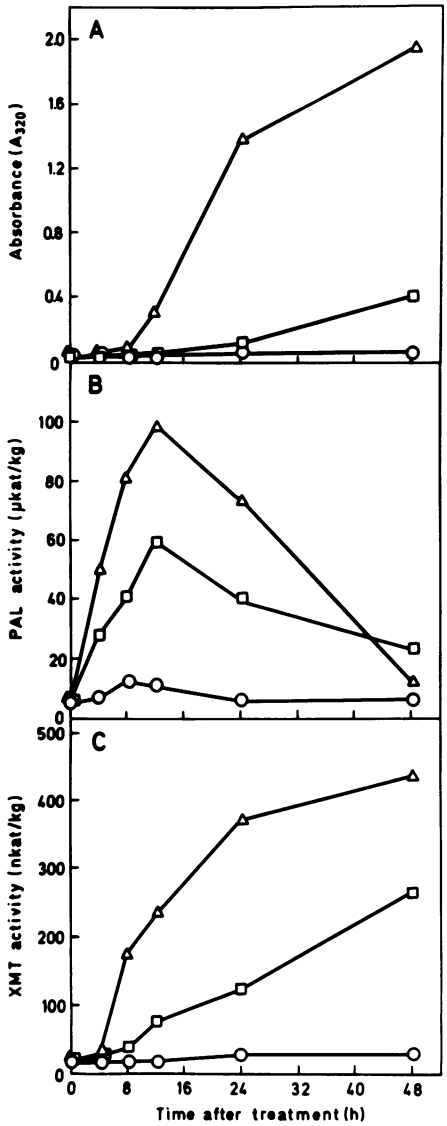

FIG. 5. Time courses for the induction of coumarins (A), PAL (B), and XMT (C) in untreated parsley cell cultures (○) and cultures treated with 10 μg/ml fungal elicitor (△) or 200 μg/ml plant elicitor II (□). Data shown are the averages of values obtained in two different experiments. PAL and XMT activities were determined as described in "Materials and Methods." Coumarins were extracted from the culture medium and quantitated as described in Figure 1.

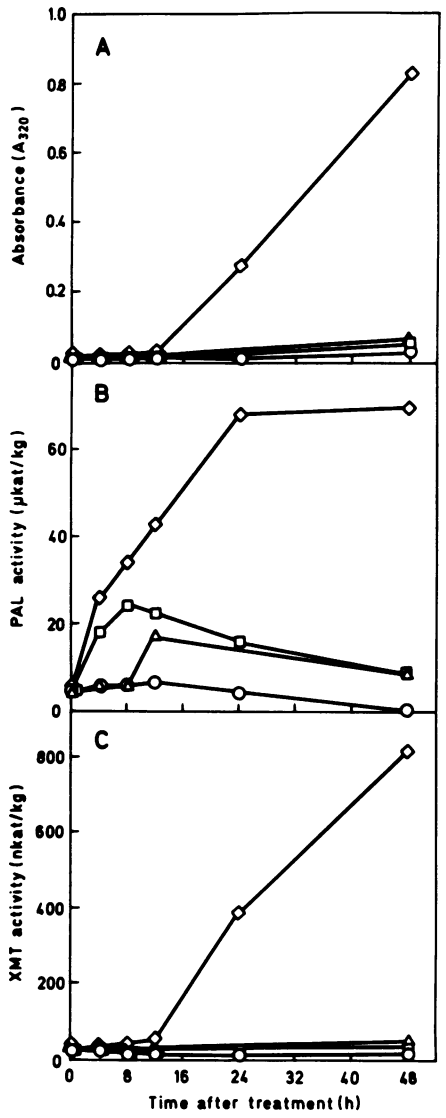

FIG. 6. Time courses for the induction of coumarins (A), PAL (B), and XMT (C) in untreated parsley cell cultures (○) and cultures treated with 0.2 μg/ml fungal elicitor (△), 100 μg/ml plant elicitor II (□), and a combination of 0.2 μg/ml fungal elicitor with 100 μg/ml plant elicitor II (◇). PAL and XMT activities were determined as described in "Materials and Methods." Coumarins were extracted from the culture medium and quantitated as described in Figure 1. A cell culture analyzed 48 h after treatment with 10 μg/ml fungal elicitor exhibited PAL and XMT activities of 14.2 μkat/kg and 450 nkat/kg, respectively; medium extracts had an A_{320} of 2.388.

present in the crude preparations. Previous studies of similar cell wall elicitors obtained by PGA lyase treatment of soybean cell walls indicated that only about 3% (w/w) of the total oligogalacturonides present in the crude preparation were elicitor-active (5). Based on these results, we estimate that the concentration of elicitor-active components present in the crude parsley cell wall elicitor preparations was below 10 μg/ml (μM concentrations) in experiments in which defense responses were significantly induced.

Analogous to the soybean system (6), we have also shown that the parsley cell wall elicitor acted synergistically with a fungal elicitor in the induction of phytoalexin accumulation in parsley cell cultures. As much as a 10-fold increase above the calculated additive response was observed in cell cultures treated with a combination of plant cell wall and fungal elicitors. Thus, the synergistic action of endogenous (plant-derived) and exogenous (pathogen-derived) elicitors appears to be a phenomenon which is not confined to soybean, where it was first observed.

The strong synergism and the similarity of responses exhibited in elicitor-treated parsley cell cultures and infected parsley tissue (W Jahnen, K Hahlbrock, manuscript in preparation) suggests that similar combinations of plant- and pathogen-derived compounds may act as elicitors during the interaction of potential pathogens with the intact plant. Our present results are an indication as to the possible nature of the signal molecules that regulate the activation of defense responses and may facilitate their identification *in situ*.

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