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Characterization of a Diffusible Signal Capable of Inducing Defense Gene Expression in Tobacco[1]

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Treatment of tobacco (*Nicotiana tabacum*) cell-suspension cultures with cryptogein, an elicitin protein from *Phytophthora cryptogea*, resulted in the release of a factor(s) that diffused through a 1000-D cutoff dialysis membrane and was capable of inducing sesquiterpene cyclase enzyme activity (a key phytoalexin biosynthetic enzyme in solanaceous plants) when added to fresh cell-suspension cultures. The diffusible factor(s) was released from cells over a 20-h period and induced a more rapid induction of cyclase enzyme activity than did direct treatment of the cultures with pure elicitin protein. The diffusible factor also induced a more rapid accumulation of transcripts encoding for sesquiterpene cyclase, acidic and basic chitinase, and *hsr203* (a putative hypersensitive response gene) than did elicitin treatment. The diffusible factor(s) was resistant to protease, pectinase, DNase, and RNase treatments, was not extractable into organic solvents, and was not immunoprecipitable when challenged with polyclonal antibodies prepared against elicitin protein. The diffusible factor(s) could not induce the release of more factor, suggesting that it was a terminal signal. These results are consistent with the notion that cells directly challenged or stimulated by pathogen-derived elicitors release diffusible secondary signal molecules that orchestrate the induction of complementary defense responses in neighboring cells.

Current models of plant-pathogen interactions envision host-specific receptors capable of recognizing elicitor-type ligands released directly or indirectly from an invading pathogen and the transduction of such recognition events into the activation of a broad repertoire of defense responses (Dixon et al., 1994; Lamb, 1994; Boller, 1995), including phytoalexin biosynthesis (Keen, 1981), synthesis and secretion of hydrolytic enzymes (Kombrink et al., 1988), rigidification of the plant cell wall (Bradley et al., 1992), and activation of a developmental program for localized cell death (Klement, 1982). Together these responses arrest the growth of the invading pathogen. Many independent lines of investigation have provided support for this model. For example, several types of elicitor molecules have been identified, including specific elicitors such as a heptaglucan elicitor that elicits phytoalexin accumulation in soybean (Sharp et al., 1984), and elicitin proteins, low-molecular-weight proteinaceous elicitors secreted by several *Phytophthora* sp. (Ricci et al., 1989) that can induce numerous plant responses such as changes in membrane permeability, alkalization of the culture medium, induction of pathogenesis-related proteins (e.g. chitinases and glucanases), and phytoalexin biosynthesis, alterations in protein phosphorylation, and the hypersensitive response (Blein et al., 1991; Milat et al., 1991; Viard et al., 1994; Tavernier et al., 1995).

The activation of defense responses has also been correlated with a variety of biochemical events that may represent components within a signal transduction cascade. These correlations have in large part been derived from two experimental approaches. The more common approach has been to use pharmacological agents known to inhibit particular biochemical processes and to examine the affects of these agents on the elicitation of specific defense response(s). Treatment of cells with antagonists for calcium-binding proteins (Vögeli et al., 1992), select protein kinase and phosphatase activity inhibitors (Grosskopf et al., 1990; MacKintosh et al., 1994), and specific ion channel blockers, for example, can prevent or delay elicitor induction of several defense responses (Renelt et al., 1993). The second approach has entailed correlating transient biochemical events such as protein phosphorylation (Felix et al., 1991) and calcium influx (Knight et al., 1991) with the induction of phytoalexin accumulation or other defense responses. Unfortunately, neither of these approaches has yielded a consensus pathway for a signal transduction cascade operating within cells to induce disease resistance. In more recent work, Levine et al. (1994) demonstrated that a rapid and transient oxidative burst preceded defense gene expression and that exogenous H_2O_2 was sufficient to induce cellular protectant genes such as GST and glutathione peroxidase and developmental changes such as pro-

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Abbreviations: CCM, control-conditioned media; ECM, elicitin-conditioned media; GST, glutathione S-transferase; MS, Murashige-Skoog.

grammed cell death. These observations led Levine et al. (1994) to propose that an oxidative (H_2O_2) burst triggered by elicitors or pathogens could serve as an important intermediary in orchestrating defense gene expression and hypersensitive cell death.

Levine et al. (1994) also suggested that H_2O_2 could serve as a diffusible intercellular signal. To test for a transmissible signal, two populations of cell-suspension cultures were separated from one another by a pair of dialysis membranes with molecular mass cutoffs of 12 and 1 kD. Inoculation of one set of cells with an avirulent pathogen induced cell death only in that population of cells in direct contact with the pathogen; cell death was not observed in the second, separated population of cells. In contrast, expression of several cellular protectant genes, including GST, was observed in both the challenged and nonchallenged populations of cells. The involvement of H_2O_2 as the diffusible signal for induction of gene expression was confirmed in experiments in which catalase was sandwiched between the two cell populations separated by the dialysis membranes. Under these conditions, GST induction was observed only in those cells in direct contact with the avirulent pathogen and not in the cells beyond the H_2O_2 trap.

Although communication between host cells in direct contact with pathogen or pathogen-derived elicitors and neighboring cells has received limited attention, results from Levine et al. (1994) and others (Dixon et al., 1983; Graham and Graham, 1994) suggest the likelihood of other diffusible signal molecules. For example, Levine et al. (1994) demonstrated that H_2O_2 was sufficient to induce antioxidant gene expression in neighboring cells but induced only weak expression of *PAL* or *CHS*, key phytoalexin biosynthetic genes in soybean. Such results raise the possibility of other diffusible molecule(s) capable of activating or potentiating expression of phytoalexin biosynthesis in adjacent cells. Graham and Graham (1994, 1996) described several such factors, referred to as competency factor(s), that are released from wounded soybean cotyledon cells and serve to enhance cellular responses when added in combination with elicitor.

To further evaluate the possibility of multiple diffusible factors released from primary-challenged cells and to extend earlier observations beyond the soybean and *Pseudomonas syringae* pv *glycinea* interaction, the current work initially sought to determine whether signal molecules sufficient for the induction of defense gene expression were released from elicitor-induced tobacco (*Nicotiana tabacum*) cell-suspension cultures. The experimental design also differed significantly from earlier studies in that a highly purified elicitin protein was used instead of an avirulent pathogen, and expression of a key enzyme for sesquiterpene phytoalexin biosynthesis (Vögeli and Chappell, 1988, 1990) was measured instead of an antioxidant gene. Similar to earlier observations with soybean, H_2O_2 does not appear to induce phytoalexin biosynthesis in tobacco. However, we report evidence for another diffusible signal that is released from tobacco cells upon interaction with the elicitin protein and is capable of inducing select defense gene expression.

MATERIALS AND METHODS

The experiments reported here have been repeated several times and data points are often averages of replicates. However, single experiments, and not averages of repeats, are shown.

Cell Cultures

Cell-suspension cultures of *Nicotiana tabacum* cv Kentucky 14 were maintained in MS medium and subcultured weekly, and their growth was monitored by measuring the increase in fresh weight (Chappell and Nable, 1987). Cultures in the rapid phase of growth (approximately 3 d after subculturing, fresh weight doubling every 2 d) were used for all experiments. Induction treatments were routinely performed in 12-well tissue culture plates with 1-mL aliquots of cell-suspension culture per well. Direct elicitor treatment was initiated by the addition of 0.1 to 1.0 μg elicitin protein mL^{-1} cell-suspension culture. The elicitin protein used in all of these studies was cryptogein, which was purified from culture filtrates of *Phytophthora cryptogea* and kindly provided by Dr. Lloyd Yu (CEPRAP, University of California, Davis). Cells were harvested by vacuum filtration and frozen in liquid nitrogen.

Diffusible Signal Assay

To assay for a diffusible signal, aliquots of tobacco cell-suspension culture were sealed inside a 1000-D molecular mass cutoff dialysis membrane (Spectrum, Houston, TX) with or without elicitin protein. The dialysis tubing was then incubated with shaking in fresh MS medium for the indicated times, and aliquots of the external medium were frozen and lyophilized immediately. The lyophilized samples were resuspended in 0.1 to 0.2 volume of sterile water relative to the volume of the initial aliquot, filter-sterilized, and used directly in the induction assays.

Sesquiterpene Cyclase Activity

Frozen cells were homogenized in 400 to 800 μL of 80 mM potassium phosphate buffer (pH 7.0), 20% glycerol, 10 mM sodium metabisulfite, 10 mM sodium ascorbate, 15 mM $MgCl_2$, and 5 mM DTT, and the slurry was centrifuged for 10 min at 12,000*g*. The cyclase assay was performed by incubating 5- to 10-μL aliquots of the supernatant (5–25 μg of protein) plus 1.5 nmol of [^{3}H]FPP (87 μCi/μmol) and sufficient reaction buffer (250 mM Tris, pH 7.0, 50 mM $MgCl_2$) to bring the final volume to 50 μL for 30 min at 37°C before extraction with 150 μL of *n*-hexane. The hexane phase was then reacted with silica powder to bind any farnesol generated by phosphatase activity. Radioactivity in an aliquot (50 μL) of the hexane phase was then determined. Sesquiterpene cyclase activity is expressed as nanomoles of cyclic product formed per hour per milligram of protein. The absolute structure of the sesquiterpene product was previously described as 5-epi-aristolochene by Whitehead et al. (1989). Enzyme assays were done in duplicate and less than 20% variation was observed between samples.

Treatment of the Diffusible Factor(s) with Hydrolytic Enzymes and Anti-Elicitin Polyclonal Antibodies

Protease type II from *Aspergillus oryzae*, pectinase from *Rhizopus* sp., RNase type II-A from bovine pancreas, and DNase I from bovine pancreas (all obtained from Sigma) were dissolved at 10 mg/mL in MS medium and then dialyzed in 1000-D cutoff dialysis tubing against the same medium for 12 h. Aliquots of CCM and ECM were subsequently incubated with each of these enzymes at 3.3 mg/mL for 2 to 4 h at 37°C. The incubation mixtures were dialyzed a second time against water, and the diffusate was collected, lyophilized, and assayed as described above.

Polyclonal antibodies to purified elicitin protein were obtained from mice using immunological procedures previously described (Vögeli et al., 1990). Purified elicitin protein was obtained by bacterial expression of a histidyl-tagged *parA1* gene from *Phytophthora parasitica* (Kamoun et al., 1993) and a single-step purification of the histidyl-tagged elicitin protein by nickel affinity chromatography (Novagen, Madison, WI). Aliquots of ECM and elicitin were incubated with polyclonal antibody serum at a 1:100 dilution in 10 mM Tris, pH 7.5, 150 mM NaCl, and 10 mM EDTA overnight at 4°C before 10 mg of protein A-agarose was added for an additional 2-h incubation at room temperature. Protein A-agarose/antibody aggregates were collected by a 5-min centrifugation and the supernatant fraction was used directly for the induction treatments described above. Treatment of the ECM and elicitin aliquots with protease XXI from *Streptomyces griseus* immobilized to agarose (Sigma) was performed similarly. Aliquots of elicitin and ECM were incubated in 10 mM Tris, pH 7.5, 150 mM NaCl, and 10 mM EDTA with 25 units of protease at 30°C for 4 h before the immobilized protease was collected by centrifugation and the supernatant fractions were used directly for induction assays.

RNA Blots

Total cellular RNA was extracted and hybridized as described by Pepper et al. (1994). Hybridization probes were tobacco sesquiterpene cyclase (Back and Chappell, 1995), acidic and basic chitinase (Linthorst et al., 1990), PR1 (Cutt et al., 1988), and *hsr203* (Pontier et al., 1994) cDNAs.

RESULTS

Characterization of the Diffusible Signal

The experimental strategy used to investigate the release of a diffusible signal(s) from elicited tobacco cells was similar to that described by Levine et al. (1994) and is shown in Figure 1. Tobacco cells with or without cryptogein, a highly purified, 10,000-D proteinaceous elicitor (Blein et al., 1991), were sealed within 1,000-D cutoff dialysis tubing and immersed in fresh growth medium. Aeration was maintained by placing the cultures on a gyratory shaker. Aliquots of the external medium were withdrawn after various incubation periods, concentrated by lyophilization, resuspended in water as 5- to 10-fold concentrates, and filter-sterilized, and an aliquot was

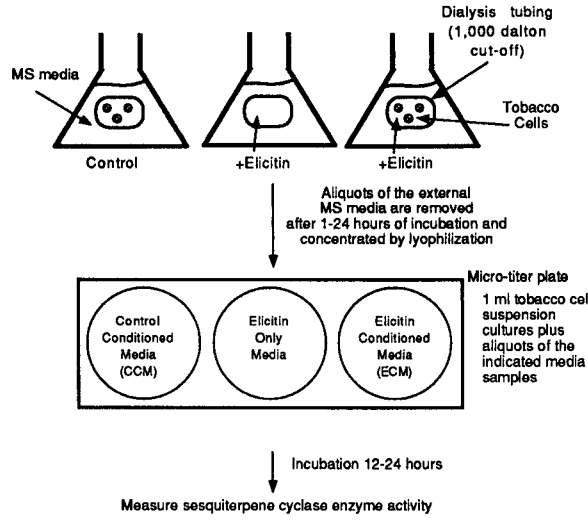


Figure 1. Bioassay for a diffusible signal capable of triggering the induction of a phytoalexin biosynthetic enzyme. Tobacco cells sealed in dialysis tubing (1,000-D cutoff) with and without cryptogein, a 10,000-D proteinaceous elicitor (Blein et al., 1991), were bathed in fresh external MS medium for various lengths of time. The external medium samples were concentrated by lyophilization and aliquots were tested for their ability to induce sesquiterpene cyclase enzyme activity (Vögeli and Chappell, 1988, 1990) in a second incubation with tobacco cell-suspension cultures.

added to rapidly growing tobacco cell-suspension cultures. These cultures were incubated 12 to 16 h before harvesting the cells and measuring extractable sesquiterpene cyclase enzyme activity.

Tobacco cells incubated without any additions contained little if any sesquiterpene cyclase enzyme activity (Table I). However, cyclase activity was induced at least 50-fold in cells treated directly with the elicitin protein. In assays for diffusible signals, CCM, the external medium resulting from incubating only tobacco cells sealed within the dialysis tubing, did not induce significant cyclase activity. In contrast, ECM, the external medium resulting from incubating tobacco cells plus elicitin protein within the dialysis tubing, induced cyclase activity to a similar extent as adding the elicitin protein directly to the cells. The induction of cyclase activity by ECM was not due to some low-molecular-weight, diffusible component within the elicitin preparation because the elicitin-only medium, the external medium resulting from incubating only an aliquot of the elicitin protein within the dialysis tubing, did not induce significant cyclase activity in the subsequent bioassay.

Induction of sesquiterpene cyclase activity in tobacco cells was dose-dependent on the ECM (Table II). Maximal induction of cyclase activity was typically observed with 200 to 400 μL of a 5-fold concentrate of the external medium. This result is based on a standardized ratio of the external medium volume to the volume of cells sealed within the dialysis tubing of 5:1 to 10:1. Release of the diffusible factor(s) from the cultures was also time-dependent, with approximately one-half of the maximum diffusible component being released within 4 h of initiating

Table I. *Bioassay for secondary signals capable of inducing sesquiterpene cyclase enzyme activity in tobacco cells*

Tobacco cell-suspension cultures were incubated with the indicated additions for 16 h before the sesquiterpene cyclase enzyme activities were determined. Conditioned media samples were prepared as follows: 2 mL of cells (CCM), 2 mL of MS media plus 0.5 μg of cryptogein, or 2 mL of cells plus 0.5 μg of cryptogein (ECM) sealed in dialysis tubing with a 1000-D cutoff were bathed in 20 mL of fresh, external MS media for 16 h. The external MS media was then concentrated 8-fold by lyophilization and 50-μL aliquots were tested for their ability to induce sesquiterpene cyclase in a second incubation with 1 mL of tobacco cell-suspension cultures.

Treatment	Enzyme Activity
	nmol mg^{-1} protein
None (control cells only, negative control)	1.3
Elicitin (0.1 μg/mL, positive control)	54.7
CCM	2.0
ECM	52.8
Elicitin-only media	4.3

the elicitin treatment (Fig. 2). A slower release of additional secondary factor(s) over the next 20 h was observed.

Treatment of the ECM with hydrolytic enzymes such as protease, pectinase, RNase, or DNase did not destroy the ability of the medium to induce cyclase activity (Table III). The diffusible factor(s) is also not likely to be H_2O_2 or a related activated oxygen species. Neither treatment of the ECM with catalase nor lyophilization of the ECM diminished the cyclase-inducing activity, although both treatments would be expected to eliminate H_2O_2 or related oxygen radicals from the ECM (data not shown). Consistent with this observation, direct addition of 100 μM to 5 mM H_2O_2 to the cell cultures did not induce cyclase activity to any appreciable extent (S. Yin and J. Chappell, unpublished data). By similar criteria, salicylic acid has also been excluded as a component of the diffusible factor(s). No

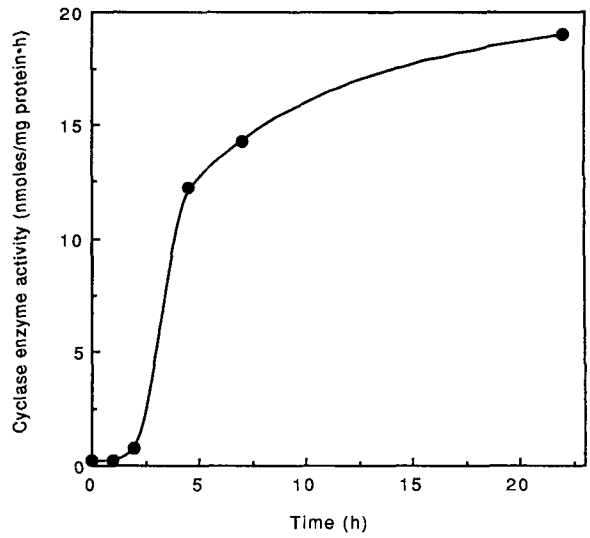


Figure 2. Time course for the release of a diffusible signal from elicitin-treated tobacco cells. Ten milliliters of tobacco cell-suspension culture plus 4 μg of cryptogein were sealed in a dialysis tubing with a 1000-D cutoff and bathed in 50 mL of fresh external MS medium. Samples of the external medium were collected at the times indicated and concentrated 6-fold by lyophilization, and 400-μL aliquots were tested for their ability to induce sesquiterpene cyclase in a second incubation with 3.5 mL of tobacco cell-suspension culture.

salicylate could be detected in the ECM using conventional chemical means such as TLC and mass spectrometry, and there was no indication of cyclase induction in cells treated with 1 to 20 mM salicylate (S. Yin, M. Lusso, and J. Chappell, unpublished data). The diffusible signal(s) is also not hydrophobic in nature since it did not partition into organic solvents such as chloroform or hexane, nor was the activity remaining in the aqueous phase altered by these treatments (data not shown). Finally, the diffusible factor(s) is not temperature-sensitive. For instance, heating of the factor(s) to 65°C for 15 min had no effect on its activity.

Table II. *Dose-dependent induction of sesquiterpene cyclase enzyme activity in tobacco cell-suspension cultures by ECM*

Tobacco cell-suspension cultures were incubated with the indicated additions for 16 h before the sesquiterpene cyclase enzyme activities were determined. Conditioned media samples were prepared as follows: 20 mL of cells (CCM) or 20 mL of cells plus 8 μg of cryptogein (ECM) sealed in dialysis tubing with a 1000-D cut-off were bathed in 50 mL of fresh, external MS media for 16 h. The external MS media samples were then concentrated 5-fold by lyophilization and aliquots were tested for their ability to induce sesquiterpene cyclase in a second incubation with 4 mL of tobacco cell-suspension cultures. Control assays included incubations of 4 mL of tobacco cell-suspension cultures with appropriate amounts of fresh MS media (MS only), and cells plus 0.5 μg mL^{-1} cryptogein and the appropriate amounts of fresh MS media (MS plus elicitin).

Aliquot	Sesquiterpene Cyclase Enzyme Activity			
	MS only	MS + elicitin	CCM	ECM
μL	*nmol h^{-1} mg^{-1} protein*			
50	2.8	36.5	0.8	3.0
200	2.0	40.9	0.8	20.8
400	2.0	42.9	1.4	31.6

Table III. *The diffusible signal is resistant to protease, pectinase, RNase, and DNase treatments*

Hydrolytic enzymes prepared at an initial concentration of 10 mg/mL were first dialyzed overnight in 1000-D cutoff tubing. Aliquots of the hydrolases corresponding to 5 mg were then incubated with 1 mL of ECM for 2 to 4 h. The ECM-hydrolase mixtures were dialyzed a second time in a 1000-D cutoff dialysis tubing against 5 mL of H_2O. The external H_2O samples were collected, lyophilized, and resuspended in a final volume of 100 μL, and entire samples were incubated with tobacco cell-suspension cultures for 16 h before the sesquiterpene cyclase enzyme activities were determined.

Treatment	Enzyme Activity
	nmol h^{-1} mg^{-1} protein
None	0.5
ECM	54.0
ECM + protease	49.4
ECM + pectinase	44.9
ECM + RNase	32.5
ECM + DNase	38.6

Although the proteolytic treatment shown in Table III suggested that the diffusible factor(s) within the ECM was not proteinaceous, such data are not sufficient to exclude this possibility. For example, the diffusible factor could be a peptide fragment released from the parent elicitin molecule by an endoproteolytic activity associated with the tobacco cells, and the released peptide fragment could be resistant to the protease treatment shown in Table III. This possibility, however, seems unlikely. If elicitin and ECM are subject to immunoprecipitation treatments with elicitin-specific polyclonal antibodies prior to their addition to the cell cultures, greater than 95% of the elicitin-inducible activity is lost and there is only a slight loss in the inducing activity of the ECM (Table IV). Additional experiments comparing the protease sensitivity of the diffusible signal(s) within the ECM with that for the elicitin protein also serve to differentiate the chemical nature of these two activities (Table IV). Greater than 80% of elicitin's inducible activity is lost upon treatment with a second type of protease, whereas the cyclase-inducing activity of the ECM fraction is largely resistant.

Is the Diffusible Factor(s) a Primary Signal?

The diffusible factor(s) could represent either a primary signal, an integral component of a signal transduction chain operating to induce the complete repertoire of defense responses, or a secondary type of signal, a component of relatively low efficiency and specificity for inducing defense responses that might be released nonspecifically from necrotizing cells. If the diffusible factor(s) was a primary signal, then the intrinsic activity of the factor(s) to induce sesquiterpene cyclase enzyme activity and other defense responses would be expected to be greater than the elicitin protein itself. To begin distinguishing between these possibilities, the induction time course of cyclase activity was determined using optimal amounts of ECM and elicitin. ECM treatment caused a significantly more rapid induction of cyclase activity than did elicitin treatment, with half-maximum activity occurring 5 to 6 h after initiation of ECM treatment and 10 or more hours required for the elicitin treatment (Fig. 3). Both ECM and elicitin treatments, however, induced cyclase activity to the same maximum by 14 to 15 h after initiation of the treatments.

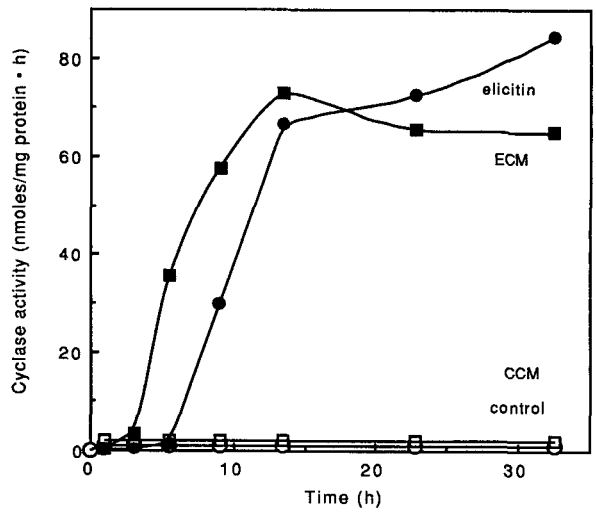


Figure 3. Comparison of the induction time course of sesquiterpene cyclase activity in tobacco cell-suspension cultures treated with elicitin directly or ECM. Enzyme activity was measured in extracts prepared from control cultures (O) and cultures receiving CCM (□), ECM (■), or elicitin (1 μg/mL, ●). Conditioned media samples were prepared and used as in Figure 2 except that 1 mL of cells was incubated with 425 μL of ECM or CCM.

The differential induction time courses of cyclase activity by ECM and elicitin also extended to steady-state measurements of the cyclase mRNA (Fig. 4A). Elicitin-induced cyclase mRNA was first observed 3 to 6 h after initiation of the treatment, and the level of cyclase mRNA appeared to accumulate throughout the 14-h experiment. In comparison, ECM treatment induced a rapid accumulation of cyclase mRNA, with the maximum occurring approximately 3 h after initiation of the treatment and declining thereafter.

Elicitor and elicitin treatments normally result in the induction of several other defense genes, including pathogenesis-related proteins and hydrolases such as chitinase and glucanase (Ryals et al., 1994). To evaluate the spectrum of defense genes induced by ECM, the induction patterns of chitinases (Linthorst et al., 1990), *PR1* (Cutt et al., 1988), and *hsr203*, a gene whose activation in tobacco is rapid, localized, and specific for incompatible plant-pathogen interactions (Pontier et al., 1994), were determined and compared with their induction patterns by elicitin treatment. ECM treatment induced both acidic and basic chitinase more rapidly than did elicitin treatment (Fig. 4, B and C), although the induction time courses for both were significantly slower relative to that for the cy-

Table IV. *The diffusible signal does not appear to be a peptide fragment derived from the elictin protein*

Elicitin and ECM were incubated with or without polyclonal antibodies to purified elicitin protein prior to immunoadsorption with protein A-agarose, or elicitin and ECM were incubated with or without protease immobilized to agarose. Supernatant fractions remaining after the immunoprecipitation treatment or centrifugation to remove the insoluble protease were incubated with tobacco cells for 16 h before the sesquiterpene cyclase enzyme activities were determined. The cyclase activity measured in control and induced cells in this particular cell line is considerably lower than in the cell lines used in the other experiments. However, the inducibility is equal to or greater than that observed in the other experiments.

Elicitor	Treatment	Enzyme Activity
		nmol h^{-1} mg^{-1} protein (%)
Control	None	<0.01
Elicitin	None	4.05 (100)
Elicitin	Protein A-agarose	6.05
Elicitin	IgG + protein A-agarose	<0.01 (<1)
ECM	None	6.52 (100)
ECM	Protein A-agarose	6.23
ECM	IgG + protein A agarose	5.50 (84)
Elicitin	None	6.26 (100)
Elicitin	Protease	1.04 (16)
ECM	None	5.95 (100)
ECM	Protease	5.51 (92)

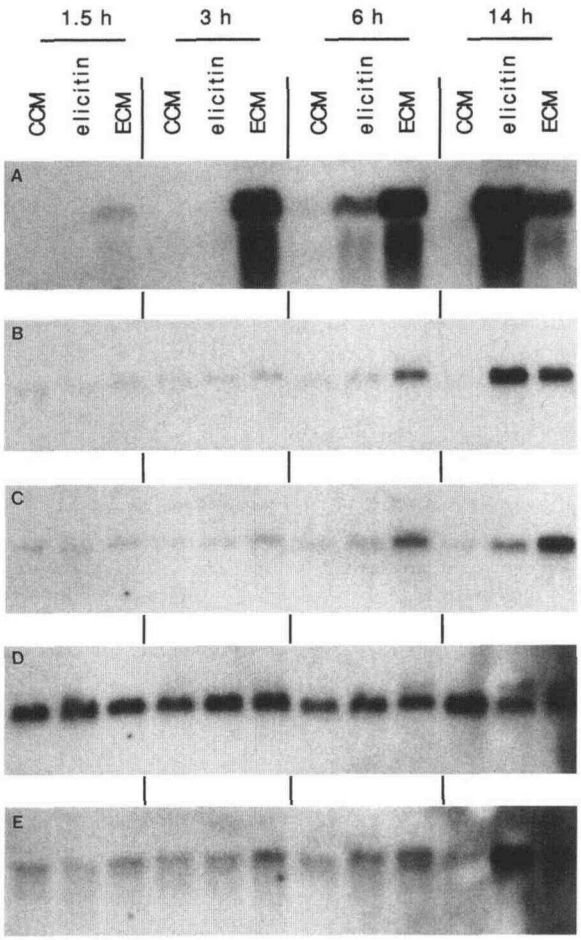


Figure 4. Comparison of the induction patterns of sesquiterpene cyclase (A), acidic (B) and basic (C) chitinase, *PR1* (D), and *hsr203* (E) mRNAs by elicitin and ECM. Total RNA was isolated from cell cultures incubated with CCM, elicitin (1 μg/mL), or ECM for the indicated lengths of time, size-fractionated by gel electrophoresis, transferred to nylon membranes, and hybridized with cDNA probes for sesquiterpene cyclase (Back and Chappell, 1995), acidic and basic chitinase (Linthorst et al., 1990), *PR1* (Cutt et al., 1988), and *hsr203* (Pontier et al., 1994) mRNAs. Conditioned media samples were prepared and used as in Figure 2, except that 1 mL of cells was incubated with 400 μL of ECM or CCM.

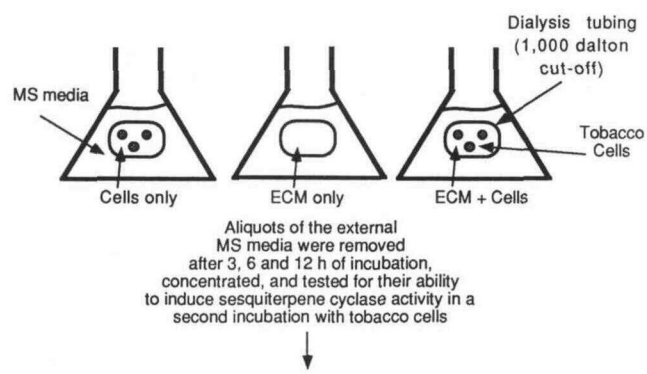
clase mRNA. *PR1* mRNA was easily detectable in control cell cultures, and there appeared to be little if any modulation of this mRNA by either the ECM or elicitin treatments (Fig. 4D). *hsr203* mRNA was also detectable in control cell cultures, and the level of this messenger tended to accumulate over the time course of this experiment in cells treated with either ECM or elicitin (Fig. 4E). However, based on the relative intensity of the hybridization signals, the ECM treatment induced a greater accumulation of *hsr203* mRNA than did elicitin treatment.

Does the Diffusible Signal(s) Regulate the Release of Additional Signal(s)?

If the diffusible signal(s) found in the ECM were to serve as a means of propagating pathogen perception to neighboring cells, then it might also stimulate the release of additional diffusible signal from cells it contacts. To evaluate this possibility, the release of diffusible factor(s) from cells sealed in 1000-D cutoff tubing with ECM were compared with the release of diffusible factor(s) from only ECM sealed in the dialysis tubing (Fig. 5). The subsequent bioassays were performed with relatively small amounts of the external medium sampled to provide for a quantitative assessment of cyclase induction by whatever diffusible signal(s) might have been released from the bagged ECM-only or ECM-plus-cells sample. Essentially all of the diffusible factor(s) was released from the ECM-only sample within the first 3 h of the experiment. A slightly lower level of diffusible signal(s) was released from the ECM-plus-cells treatment within this time frame. This might be expected if a small percentage of the diffusible factor(s) within the ECM bound to the cells within this initial period. However, no additional diffusible factor(s) was detected in the ECM-plus-cells samples at the later times, indicating that the diffusible signal(s) does not itself induce the release of more signal.

DISCUSSION

Early models describing plant-pathogen interactions relied heavily on the work of Flor (1942) and his proposal for a gene-for-gene interaction. This hypothesis has also been interpreted to mean that plant-pathogen interactions are mediated by pathogen-derived factors (elicitors) and plant



Time aliquots of media removed (h)	Sesquiterpene cyclase enzyme activity (nmol/h • mg prot)		
	Cells only	ECM only	ECM + Cells
3	0.5	6.6	5.5
6	0.9	7.6	5.6
12	0.5	5.8	5.2

Figure 5. ECM does not regulate the production of a more diffusible signal. MS or ECM samples (5 mL), with or without 5 mL of tobacco cell-suspension cultures, sealed in dialysis tubing (1000-D cutoff) were bathed in 25 mL of fresh external MS medium. One-milliliter aliquots of the external medium were removed after 3, 6, and 12 h of incubation and concentrated by lyophilization, and half of each resuspended sample was tested for its ability to induce sesquiterpene cyclase in a second incubation with 1 mL of tobacco cell-suspension culture. prot, Protein.

cell receptors and that a recognition event is followed by a signal transduction cascade, resulting in the activation of a broad repertoire of defense responses. Recently, there has been impressive confirmation of many of the components envisioned within this model. Cultivar-specific elicitors from fungal pathogens have been characterized (Scholtens-Toma and de Wit, 1988). Several disease-resistance genes have been cloned and the putative proteins encoded by these genes contain potential ligand or elicitor-binding sites (Staskawicz et al., 1995). Finally, a number of potential intracellular signal molecules mediating the activation of defense gene expression have been identified (Felix et al., 1991; Knight et al., 1991).

While compelling evidence for this "simple" model of plant-pathogen interactions has accumulated, other observations have suggested that plant-pathogen interactions are anything but simple and that there are likely to be other diffusible factors or signals that activate responses in neighboring to distant cells and contribute to a disease defense response. The induction of systemic acquired resistance via the localized synthesis and translocation of salicylate or some other signal(s) is a notable example of a long-distance communication signal (Ryals et al., 1994). The type of factor relevant to the current work, however, is one that is likely to be released quickly from the primary-challenged cells, to diffuse rapidly over short distances, and to selectively activate defense responses in cells neighboring those actually in contact with the pathogen or elicitor. Diffusible signals of this type should also be terminal signals not capable of inducing the production of more diffusible signal. Otherwise, the release of any signal molecule would be expected to self-propagate a response throughout the entire plant.

The diffusible factor(s) characterized in the current study satisfies several of these criteria. The fact that approximately 50% of the factor(s) is released within 4 h of the initial elicitor treatment and the small size of the factor (less than 1000 D) are consistent with its ability to rapidly diffuse between cells. The diffusible factor(s) from tobacco also induces rapid and selective expression of several genes representing a broad battery of defense responses. For example, the diffusible factor(s) shifts the induction pattern for sesquiterpene cyclase, acidic and basic chitinase, and *Hsr203* transcript levels 3 to 5 h earlier than elicitor treatment alone but does not alter *PR1* gene expression beyond that already evident in control cells. Sesquiterpene cyclase is a key regulatory enzyme for phytoalexin biosynthesis in solanaceous plants (Vögeli and Chappell, 1988), chitinases are secreted hydrolytic enzymes known to inhibit microbial growth (Kombrink et al., 1988), and *hsr203* is a gene whose expression is tightly correlated with the hypersensitive response (Pontier et al., 1994). The tobacco-diffusible factor(s) is also a terminal signal molecule that does not induce the release or production of more factor(s).

There have been several reports of diffusible or transmissible signals involved in orchestrating plant defense responses. Dixon et al. (1983) reported that denatured RNase, an abiotic elicitor, was able to induce the release of a low-molecular-weight soluble factor capable of inducing phenylpropanoid biosynthetic enzymes and phytoalexin accumulation in French bean hypocotyls and cell-suspension cultures. These investigators also reported that a biotic elicitor derived from fungal cell wall hydrolysates, although able to induce phenylpropanoid enzymes and phytoalexin accumulation itself, did not induce the generation of a diffusible or transmissible signal. Dixon et al. (1983) suggested that this differential response to biotic and abiotic elicitors may reflect different response mechanisms to the two different types of elicitors. For example, the abiotic elicitor used in that study was known to cause indiscriminate cell damage, which could result in the release of a compartmentalized metabolite(s) capable of inducing phytoalexin accumulation in neighboring cells. On the other hand, Graham and Graham (1994, 1996) reported transmissible signals that they referred to as elicitation competency factors, which are apparently released from wounded and possibly hypersensitive responding cells and serve to enhance the response of proximal or neighboring cells to subsequent elicitor treatment. When added in combination with cell wall glucan-type elicitor to newly cut, extensively washed cotyledons devoid of endogenous competency factors, the competency factors augmented the phytoalexin biosynthetic response. The competency factors by themselves, however, were not sufficient to induce phytoalexin biosynthesis. The diffusible factor(s) described in the current work is distinctly different from the competency factors. The factor(s) released from tobacco cells is in response to a specific proteinaceous elicitor and induces the expression of a very specific repertoire of defense genes without other exogenous factors/elicitors.

More recently and in contrast to the competency factors, Levine et al. (1994) demonstrated that H_2O_2 released from soybean cells challenged with elicitors or bacterial pathogens was sufficient to trigger expression of antioxidant gene expression in neighboring cells. The diffusible factor(s) released from tobacco cells is not likely to be H_2O_2 or another activated oxygen species (Mehdy, 1994), since these compounds would not survive the lyophilization treatment used in this study. Furthermore, exogenous H_2O_2 does not induce sesquiterpene cyclase enzyme activity to any appreciable extent (S. Yin and J. Chappell, unpublished data). Likewise, salicylic acid has been excluded as a possible component of the diffusible factor(s) because no salicylate could be detected within the ECM fraction and because salicylate alone does not induce cyclase activity (S. Yin, M. Lusso, and J. Chappell, unpublished data).

It is unlikely that the diffusible factor(s) described here is a peptide fragment released from the elicitin protein. Precedence for the release of biologically active peptides has been provided by a study that demonstrated that a precursor protein served as the substrate for the release of a peptide fragment capable of inducing selective gene expression in wounded tomato tissues (Pearce et al., 1991). More recently, Nürnberger et al. (1994) demonstrated that the inducing activity of a 30,000-D proteinaceous elicitor resides within a 13-amino-acid peptide fragment. This latter study, however, did not establish a need for an endo-

proteolytic release of the 13-residue peptide as a prerequisite for the protein's eliciting activity. Two types of evidence suggest that the diffusible factor(s) in the current study is also not derived from a selective proteolysis of the elicitin protein: (a) The diffusible factor(s) is resistant to protease digestions, which inactivates the elicitin protein, and (b) the diffusible factor(s) is not susceptible to immunoprecipitation with elicitin-specific antibodies. Although this type of evidence implies that the diffusible factor(s) does not arise as a breakdown product of the elicitin protein, it also does not rule out the possibility that induced cells could release a very discrete peptide fragment from the elicitin protein, which spontaneously folds into a protease-resistant form and a form that is not recognized by the polyclonal antibodies.

Z.-J. Guo, C. Lamb, and D.A. Dixon (unpublished data) observed results similar to those reported here. These investigators have characterized diffusible factors released from elicitor-treated soybean, alfalfa, and tobacco, and demonstrated that the factors from all three cell cultures are capable of inducing Phe ammonia lyase in all three species and phytoalexin accumulation in only soybean and alfalfa. For the diffusible signal best characterized in that study, the soybean factor, like the tobacco factor, is a low-molecular-weight compound(s) that induces select defense responses in soybean cells. The soybean factor differs from the tobacco signal in that it is composed of multiple components that can be separated by ion-exchange chromatography; at least one component of the soybean factor is also sensitive to protease treatments.

Altogether, the combined observations of Dixon et al. (1983), Graham and Graham (1994), Levine et al. (1994), Z.-J. Guo, C. Lamb, and D.A. Dixon (unpublished data), and the results of the present study suggest that cells in direct contact with pathogens may be induced to secrete a variety of signals that activate defense responses in neighboring cells. The actual contribution of these factors to a resistance response, however, is unclear. Identification of the factors, further characterization of their temporal and spatial changes during plant-pathogen interactions, and, most critically, the development of genetic mutants lacking one to several of these factors are necessary before their intrinsic contributions can be assessed.

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