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Early Changes in Gene Expression in Tobacco Cells Elicited with Cryptogein

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Isolated tobacco cells were used to examine early changes in gene expression induced by elicitation with the proteinaceous elicitor cryptogein. A two-dimensional electrophoresis strategy was carried out for the identification of marker polypeptides of elicitation. After 30 min of elicitation, 20 polypeptides appeared or increased in intensity, whereas the amounts of three other polypeptides decreased. Two-dimensional electrophoresis patterns obtained after mRNA translation in vitro revealed that the synthesis of about eight polypeptides could be regulated by the level of mRNAs. The accumulation of mRNAs encoding several known plant proteins was further examined by Northern and slot blot hybridizations. The results indicate (i) a significant transitory accumulation of mRNA encoding plasma membrane H^+-ATPase (EC 3.6.1.35), between 5 and 30 min after the addition of the elicitor, (ii) a fast and strong accumulation of mRNA encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34), and (iii) a slow accumulation of mRNA encoding phenylalanine ammonia lyase (EC 4.3.1.5) and the pathogenesis-related protein PR-b1. These results indicate that early changes in gene expression occur in the elicitation process and concern key plant enzymes.

Additional keywords: defense genes.

Nous avons utilisé des cellules de tabac en culture pour étudier les modifications précoces de l'expression de gènes provoquées par l'élicitation par un éliciteur protéique de réactions de défense, la cryptogéine. Une stratégie d'électrophorèses bidimensionnelles a tout d'abord été utilisée pour identifier des polypeptides marqueurs de l'élicitation. Après 30 min d'élicitation, 20 polypeptides apparaissent ou ont leur synthèse augmentée tandis que la synthèse de trois autres polypeptides est diminuée. Les diagrammes d'électrophorèse obtenus après traduction in vitro montrent que la synthèse d'au moins huit polypeptides pourrait être régulée au niveau transcriptionnel. L'accumulation d'ARN messagers codant pour des protéines connues de plantes a ensuite été étudiée par hybridations de Northern-blots. Les résultats montrent (i) une accumulation transitoire d'ARN messagers codant pour l'H^+-ATPase de la membrane plasmique (EC 3.6.1.35), entre 5 et 30 minutes après l'addition de l'éliciteur, (ii) une augmentation rapide et importante de l'accumulation d'ARN messagers codant pour la 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34), et (iii) une accumulation plus lente des ARN messagers codant pour la phenylalanine ammonia lyase (EC 4. 3.1.5) et pour une protéine PR, la PR-b1. Ces résultats indiquent qu'il se produit de nombreuses modifications précoces de l'expression de gènes au cours de l'élicitation et que certaines de ces modifications concernent des enzymes clés du métabolisme végétal.

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Plants respond to pathogen aggression by activating a variety of defense reactions. Many of these responses, such as the accumulation of phytoalexins and pathogenesis-related (PR) proteins, are initiated by gene activation (Darvill and Albersheim 1984; Bowles 1990; Dixon and Lamb 1990). Experimental models employing cultured plant cells and elicitor preparations from pathogens have proven to be good tools for the identification and isolation of putative defense-related genes, such as genes encoding for chalcone synthase and chalcone isomerase (Hahlbrock and Scheel 1989) and phenylalanine ammonia lyase (PAL) (Habereder et al. 1989) and still unidentified genes (Schmelzer et al. 1989). The elicitor-active molecules are chemically diverse. Several microbial and fungal proteins, glycoproteins (Billard et al. 1988; Ricci et al. 1989), or oligosaccharides of either plant or fungal cell wall polysaccharides (for review see Dixon and Harrison 1990; Ryan and Farmer 1991; Darvill et al. 1992) with elicitor activity have been characterized.

Ricci and coworkers (Ricci et al. 1989) purified a 10-kDa protein named cryptogein from a culture filtrate of *Phytophthora cryptogea.* Cryptogein causes hypersensitive reaction (HR)–like necrosis in tobacco (*Nicotiana tabacum*) at the site of application (stem or roots) and also on distant leaves. Treated tobacco plants acquire resistance to subsequent inoculation with the tobacco pathogen *Phytophthora parasitica* var. *nicotianae* (Ricci et al. 1989).

Cryptogein is an elicitor of defense reactions, including the production of ethylene, phytoalexins, and PR proteins in tobacco plants and also in cultured cells (Bonnet et al. 1986; Milat et al. 1990; Blein et al. 1991). These defense responses, which are detected relatively late (2 to 4 h after treatment), probably result from the activation of specific genes. The ear-

liest known responses to cryptogein in cell suspensions are rapid increases in pH and in the conductivity of the extracellular medium without affecting the integrity of the plasma membrane (Blein et al. 1991). These responses are inhibited by staurosporine, an inhibitor of protein kinases. Cryptogein induces a staurosporine-sensitive phosphorylation of several polypeptides (Viard et al. 1994). These observations suggest that phosphorylated proteins may be essential for the transduction of elicitor signals (Felix et al. 1991). In this work, we investigated the earliest changes induced by cryptogein in gene expression of tobacco cells. First, we used a two-dimensional electrophoresis strategy to identify marker polypeptides of cryptogein action. We analyzed the polypeptides produced in vivo in untreated and cryptogein-elicited tobacco cells and polypeptides obtained after in vitro translation of mRNA purified from the same cells. In a second step, we performed Northern and slot blot analysis to examine the kinetics of accumulation of mRNAs encoding for enzymes probably involved in defense reactions in plants, such as PAL, *O*-methyltransferase (OMT) (EC 2.1.1.6), the PR protein PR-b1, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and for a plasma membrane H^+-ATPase. Plasmalemma H^+-ATPase was investigated because some of the effects of cryptogein (medium alkalinization and K^+ efflux) are opposite those of fusicoccin, which stimulates this enzyme (Blein et al. 1991).

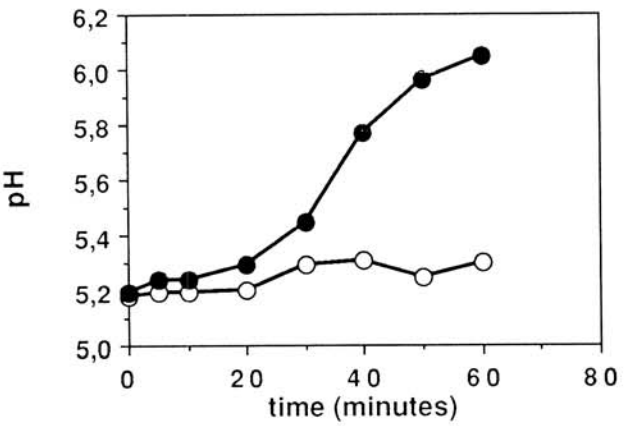

Fig. 1. Time course of changes in pH of tobacco cell suspensions elicited with 10 nM cryptogein (●) and control cell suspensions (○).

RESULTS

Response of tobacco cells to cryptogein: extracellular alkalinization.

A strong alkalinization of the culture medium was induced rapidly after cryptogein addition. This effect reached a maximum within 60 min (Fig. 1). These observations are in accordance with previous results (Blein et al. 1991; Viard et al. 1994). For these authors the alkalinization of the extracellular medium after cryptogein treatment could be considered a good marker of elicitation. Following these kinetics, protein and RNA analyses were carried out on cells harvested 5 min after treatment (before the start of alkalinization), 30 min after treatment (at the beginning of the exponential phase of alkalinization), 60 min after treatment (at the beginning of the plateau), and 3 h after treatment. The same analyses were carried out on control cells harvested at the same times.

Changes in two-dimensional polypeptide patterns during cell elicitation.

In vivo polypeptides. Analysis of two-dimensional protein patterns was chosen to identify specific markers of cryptogein elicitation. The densitometric quantification of spots provided an indication of the relative variation of each polypeptide. The variability in spot density was corrected by using a battery of constant spots as internal standards and a Carbamalyte kit (Pharmacia) as external standards. This method allowed a

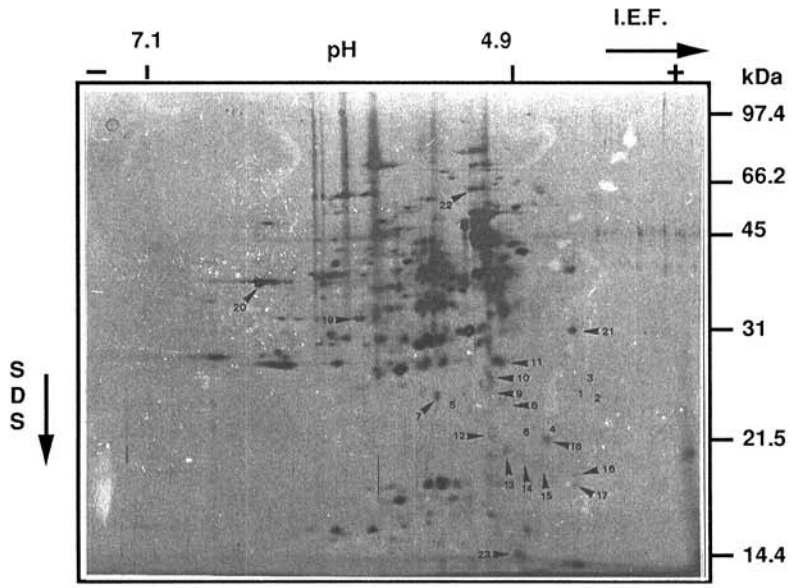

Fig. 2. Two-dimensional electrophoresis pattern of total proteins extracted from control cells: 20 µg of total protein was separated by isoelectric focusing (IEF) in the first dimension and by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis in the second dimension. The arrows and numbers indicate spots found to vary or the locations of spots present at various times after elicitation with 10 nM cryptogein.

kinetic analysis of polypeptide accumulation after cryptogein addition.

Figure 2 shows about 500 silver-stained polypeptides in control cells. About 23 spots varied during cryptogein treatment. Each polypeptide was characterized by its isoelectric point and its molecular mass (Table 1).

In cryptogein-treated cells, the accumulation of 20 polypeptides (1–20) was enhanced, whereas the levels of three polypeptides (21–23) were lowered (Fig. 3). In order to check whether some cryptogein-modulated polypeptides were specific, polypeptides induced during the HR in tobacco leaves were located in two-dimensional gel patterns (Oulad Abdelghani et al. 1991). A comparison of polypeptides from healthy tobacco leaves with those from leaves of plants infected with tobacco mosaic virus revealed a group of polypeptides specific for the HR to the virus. Only one of these polypeptides presented the same characteristics in the two patterns (polypeptide 12, pI 5.5, 22 kDa).

Relative variations in these spots during the treatment were quantified for each cell sample (Fig. 3). Polypeptide spots were divided into six groups (a–f) according to the kinetics of accumulation.

Some polypeptides (1–6) were undetectable in control cells but accumulated 30 to 60 min after the addition of cryptogein (Fig. 3A). Other polypeptides (8–10), which were present in small amounts in control cells, increased greatly within 30 min after the addition of the elicitor (Fig. 3B), whereas polypeptides 7, 11, and 13–18, which were present in control cells in greater amounts, increased more slowly after the addition of the elicitor (Fig. 3C). Polypeptide 12 accumulated to a high level 30 min after the start of elicitation (Fig. 3D), but it had also started to accumulate in control cells after 60 min. Polypeptides 19 and 20, present in large amounts in control cells, increased slightly in elicited cells (Fig. 3E). Polypeptides 21–23 decreased greatly within 60 min after the start of elicitation (Fig. 3F). All these polypeptides could be considered early markers of cryptogein action.

Table 1. Characterization of polypeptides whose levels are affected by cryptogein treatment

Polypeptide	Kinetic group[a]	pI	Molecular mass (kDa)
1	a	5	24
2	a	4.9	22
3	a	4.9	25
4	a	5.15	21.5
5	a	5.66	22
6	a	5.34	21.5
7	c	5.74	24
8	b	5.46	23
9	b	5.39	24.5
10	b	5.39	26
11	c	5.37	27.5
12	e	5.48	20
13	c	5.4	21
14	c	5.3	16.5
15	c	5.18	16
16	c	5	18
17	c	5.05	16.5
18	c	5.13	20
19	d	5.85	34
20	d	6.57	39.5
21	f	4.9	32
22	f	5.56	65
23	f	5.35	14.5

[a] Polypeptides are put in six groups (a–f) according to the kinetics of their accumulation (see Fig. 3).

In vitro–translatable mRNAs. In vitro translation of mRNAs obtained from control or elicited cells was performed in order to measure the effects of elicitation on transcription. About 15 polypeptides were affected by cryptogein treatment (Fig. 4). Five polypeptides appeared after 5 min of treatment, and the others between 5 and 60 min. These results suggested that cryptogein could modulate the accumulation of some polypeptides by rapidly changing the steady-state levels of the corresponding mRNAs. It is difficult to compare the two-dimensional patterns obtained after in vitro translation with the two-dimensional patterns obtained from total proteins in vivo, because some mRNAs could be only partly translated, and the polypeptides translated in vitro were not mature. Nevertheless, comparisons of molecular masses and isoelectric points suggest that the synthesis of polypeptides 14, 16, 20, and 23 could be modulated at the transcriptional level, but only microsequencing would allow the identification of these polypeptides.

Effects of cryptogein on steady-state levels of mRNAs for H^+-ATPase, HMGR, PAL, OMT, and PR-b1.

RNAs encoding plasma membrane H^+-ATPase, HMGR, PAL, OMT, and PR-b1 were detected and quantified by Northern blot and slot blot hybridizations in total mRNA preparations (Fig. 5). The integrity of the RNA preparations was checked by electrophoresis of total RNA samples in the presence of ethidium bromide. Quantitation was carried out on slot blots, but all the results were confirmed by Northern blot analysis (not shown).

A significant accumulation of mRNA encoding plasma membrane H^+-ATPase occurred between 5 and 30 min after the addition of cryptogein, after which the mRNA returned to its initial level (Fig. 5A). mRNA encoding HMGR started to accumulate rapidly between 5 and 30 min after the addition of the elicitor (Fig. 5B). Hybridization with clone 235, a cDNA probe corresponding to a gene activated during a tobacco-*Pseudomonas* interaction (Marco et al. 1990), showed a large accumulation of the corresponding mRNA between 30 and 60 min after elicitation (Fig. 5C). RNA encoding PAL accumulated slowly and slightly during the treatment (Fig. 5D). RNA encoding OMT, which had a high initial level, increased slowly 60 min after the start of elicitation (Fig. 5E). Used as a control, mRNA encoding PR-b1 cDNA showed a slight increase after 3 h of elicitation, but a slight increase was also noticed in the control cells (Fig. 5F).

DISCUSSION

The aim of our study was to explore the earliest modulations in gene expression following the addition of the elicitor. Two-dimensional electrophoresis revealed that some polypeptides started to accumulate 30 min after the addition of the elicitor. It is interesting to note that other polypeptides disappeared at the same time. All these polypeptides, which could be used as markers of elicitation with cryptogein, will have to be sequenced to obtain further information about their function. However, the results show that cryptogein rapidly modulates, positively or negatively, the net synthesis of some

polypeptides. For some polypeptides the modulation could happen at the transcriptional level.

Elicitation with cryptogein induces the accumulation of transcripts corresponding to particular genes with various temporal patterns of accumulation. The earliest event observed is the accumulation of transcripts for plasma membrane H^+-ATPase. This accumulation occurs after 5 min but is transitory: 30 min after the start of elicitation the plasma membrane H^+-ATPase transcript returns to its initial level. Thus, the inhibition of the plasma membrane H^+-ATPase, which could explain the alkalinization of the extracellular medium and the K^+ efflux in treated cells, may lead to a transient increase in the H^+-ATPase transcript level before degradation. Other mechanisms could be involved in ATPase inactivation, such as phosphorylation-dephosphorylation (Vera-Estrella et al. 1994).

As shown in Figure 5B, mRNA encoding HMGR accumulates rapidly and in great amounts after the addition of cryptogein. The corresponding HMGR gene was previously found to be activated in tuber potato tissues after wounding and after treatment with arachidonic acid (Choi et al. 1992; Choi and Bostock 1994); in *Nicotiana sylvestris* protoplasts, roots, and leaves 12 to 24 h after various stresses, such as viral infection or $HgCl_2$ treatment (Genschick et al. 1992); and in cultured cells of *Calypogeia granulata* elicited by vanadate (Nakagawara et al. 1993). This gene could be considered a defense gene. HMGR catalyzes the first step in isoprenoid biosynthesis by converting 3-hydroxy-3-methylglutaryl–coenzyme A to mevalonic acid (Bach 1987), which is the basic precursor of all cellular isoprenoids, including sesquiterpene phytoalexins. Thus, the fast activation of the transcriptional activity of the HMGR gene during cryptogein elicitation may be one important event in the HR.

Our results also show a strong accumulation of mRNA corresponding to clone 235 between 30 and 60 min after treatment. However, this accumulation occurs later than those of HMGR-encoding mRNAs. Clone 235 belongs to a group of cDNA clones corresponding to mRNAs that accumulate during the HR of tobacco leaves infiltrated with an incompatible strain of the bacterial pathogen *Pseudomonas solanacearum.* (Marco et al. 1990). Godiard and coworkers (1991) showed an accumulation of mRNA corresponding to clone 235 in tobacco cells treated with an elicitor isolated from *Phytophthora parasitica* var. *nicotianae.* This accumulation began in

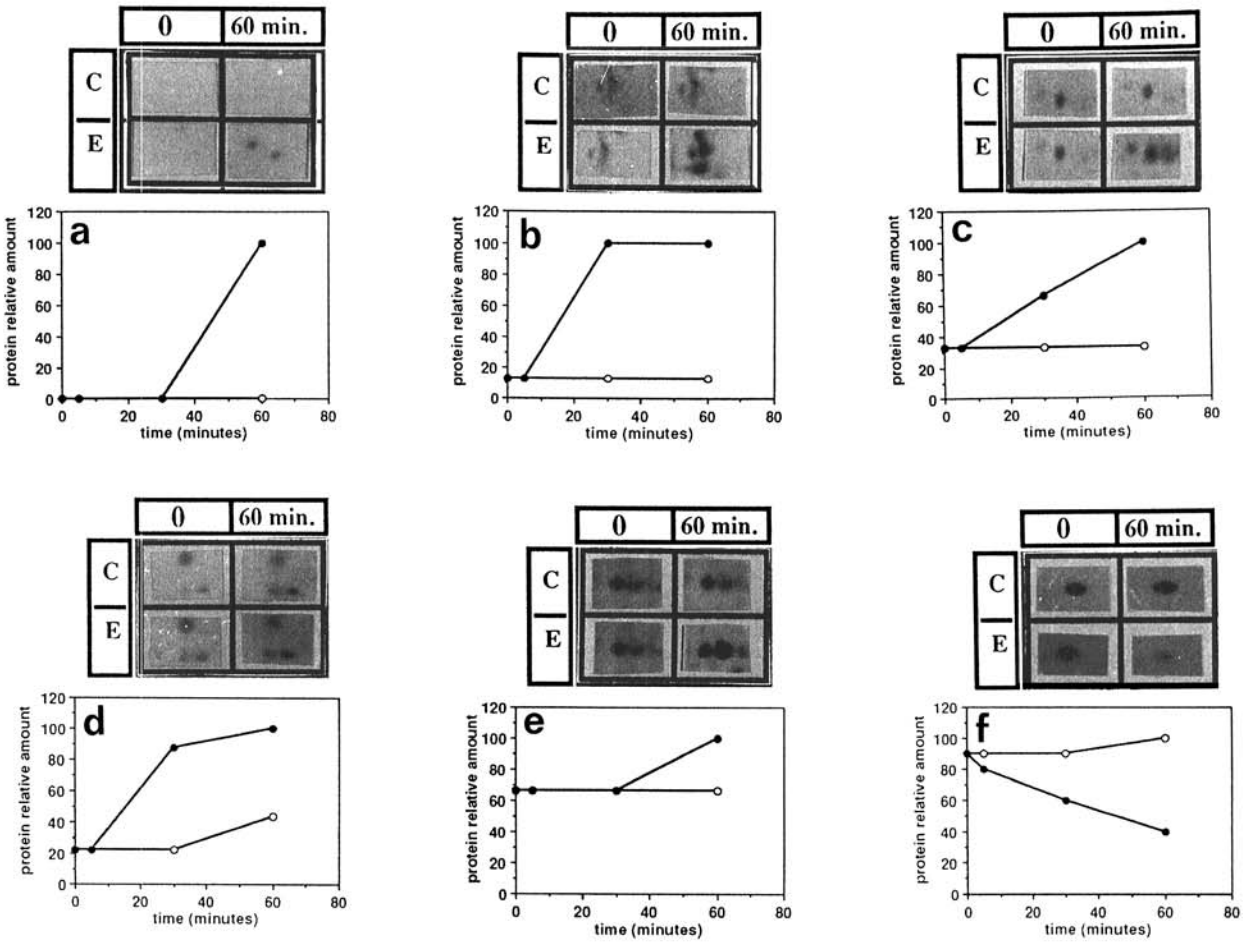

Fig. 3. Time course of changes in polypeptide content of elicited (E) tobacco cells after treatment with 10 nM cryptogein (●) and control (C) tobacco cells (○). Polypeptides were detected as silver-stained spots on two-dimensional gels (for the locations of the spots, see Fig. 2). The relative amount of each polypeptide was estimated by image analysis as described in Materials and Methods. Each graph presents the kinetics of accumulation of one polypeptide representative of one of the groups a–f: spots 1 and 2 (**A**), spots 8–10 (**B**), spots 13–15 (**C**), spot 12 (**D**), spot 19 (**E**), and spot 21 (**F**). The inserts show the corresponding spots from two-dimensional maps. For characteristics of groups a–f and spots, see Table 1.

treated cells after 50 min of treatment, as was observed with cryptogein. No variations were shown in the accumulation of OMT mRNA.

We used PAL and PR-b1 probes as controls. PAL is a key regulatory enzyme of phenylpropanoid metabolism, catalyzing the first reaction leading to a number of potentially protective compounds, such as flavonoids. This enzyme is highly regulated during development and in response to a range of environmental factors, including wounding, infection, light, phytohormones, and elicitors (Dixon et al. 1983; Jones 1984). RNA blot hybridizations and nuclear runoff experiments have shown that yeast and fungal elicitors rapidly stimulate transcription of PAL genes, leading to marked accumulation of PAL mRNA and hence increased enzyme synthesis and activity (Edwards et al. 1985; Gowri et al. 1991). We observed an increase in the accumulation of PAL mRNA between 30 and 60 min after the addition of cryptogein. This increase is slightly lower and slower than the increase in HMGR mRNA.

It has been reported that PR proteins are produced during the response of plants to infection by viruses, viroids, bacteria, and fungi (for reviews, see Van Loon 1985; Stintzi et al. 1993). The level of mRNA for PR-b1 was reported to increase over 100-fold 2 days after inoculation with tobacco mosaic virus (Hooft von Huijsduijnen et al. 1985). Our study indicates a slight accumulation of PR-b1 mRNA after 3 h in cells treated with cryptogein, compared to the control cells. Thus PR proteins may be produced in tobacco cells treated with cryptogein, as was previously reported in tobacco plants (Bonnet et al. 1986).

These results confirm that cell suspensions could be a good tool for investigating elicitor action. This tool made it possible to detect transcriptional activation of identified genes (plasma membrane ATPase and HMGR) within a few minutes and the identification of polypeptides which appeared or disappeared in the first 30 min of elicitation.

A more precise investigation of gene activation and inactivation at the early stages of cryptogein elicitation is now in progress in our laboratory, using a modified differential display reverse transcriptase polymerase chain reaction.

MATERIALS AND METHODS

Materials.

Cell suspensions of tobacco (*Nicotiana tabacum* cv. Xanthi) were grown at 25°C under continuous light of low intensity (photon flux rate of 30 to 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in Chandler's medium (Chandler et al. 1972) on a rotary shaker (150 rpm) and subcultured every 5 days in order to maintain the culture in exponential growth phase.

Cryptogein was purified according to Ricci et al. (1989) and added to the suspension cultures as an aqueous solution.

Extracellular pH measurement.

Cells in exponential growth phase were harvested by filtration, washed, and resuspended (0.1 g/ml, fresh weight) in 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 2.0 mM morpholinoethanesulfonic acid buffer, pH 5.75 (Blein et al. 1991). Aliquots of this suspension were preincubated for 4 h (150 rpm, 25°C, light) before the addition of 10 nM cryptogein. Extracellular pH was monitored in control cell suspensions and in cell suspensions treated with cryptogein. At determined times (5, 10, 15, 20, 30, 45, and 60 min) cells were collected by filtration, washed, frozen in liquid nitrogen, and stored at –80°C. All manipulations were carried out under sterile conditions.

Protein extraction and assays.

Cells were ground in liquid nitrogen. The powder obtained was resuspended for 1 h at –20°C in 10% (wt/vol) trichloroacetic acid in acetone containing 0.07% (vol/vol) 2-mercapto-

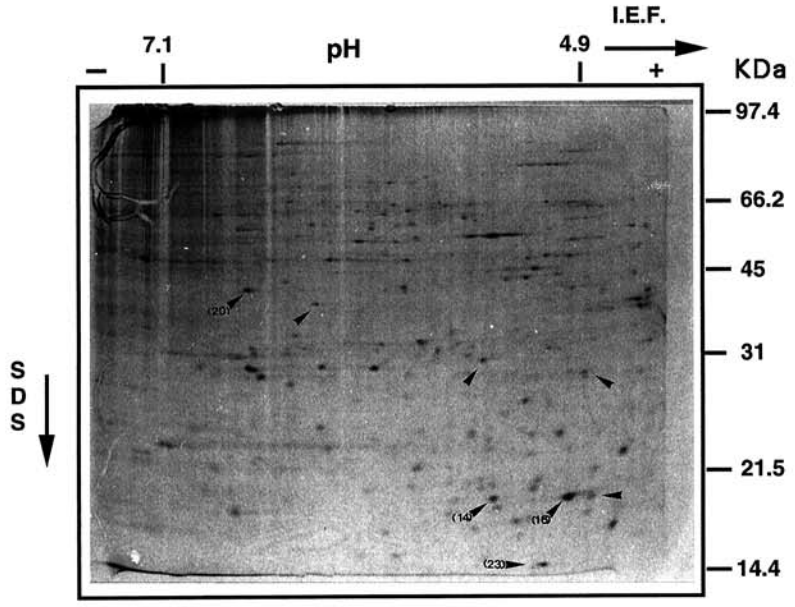

Fig. 4. Two-dimensional electrophoresis pattern of polypeptides obtained after in vitro translation of mRNAs extracted from cells harvested after 60 min of cryptogein elicitation. The arrows indicate spots found to vary as a function of cryptogein supply. The numbers in brackets indicate spots previously located at the same isoelectric point and molecular weight (Fig. 2).

ethanol. These crude extracts were centrifuged at 12,000 × *g* for 30 min. The pellet was rinsed in cold acetone for 1 h at −20°C and then centrifuged at 12,000 × *g* for 20 min), vacuum-desiccated, and dissolved in electrophoresis buffer, consisting of 9.5 M urea, 2% (wt/vol) Nonidet P40, 5% mercaptoethanol, and 3.2% ampholines 3–10 (Pharmacia).

Total protein was estimated in pelleted crude extracts according to Bradford (1976) and directly in electrophoresis samples with a modified Bradford assay (Ramagli and Rodriguez 1985).

Protein two-dimensional electrophoresis.

Electrophoresis was carried out in a Miniprotean II 2-D cell (Bio-Rad). The first dimension was isoelectric focusing, performed according to O'Farrell (1975), with minor modifications. The first-dimension gel, consisting of 4% (wt/vol)

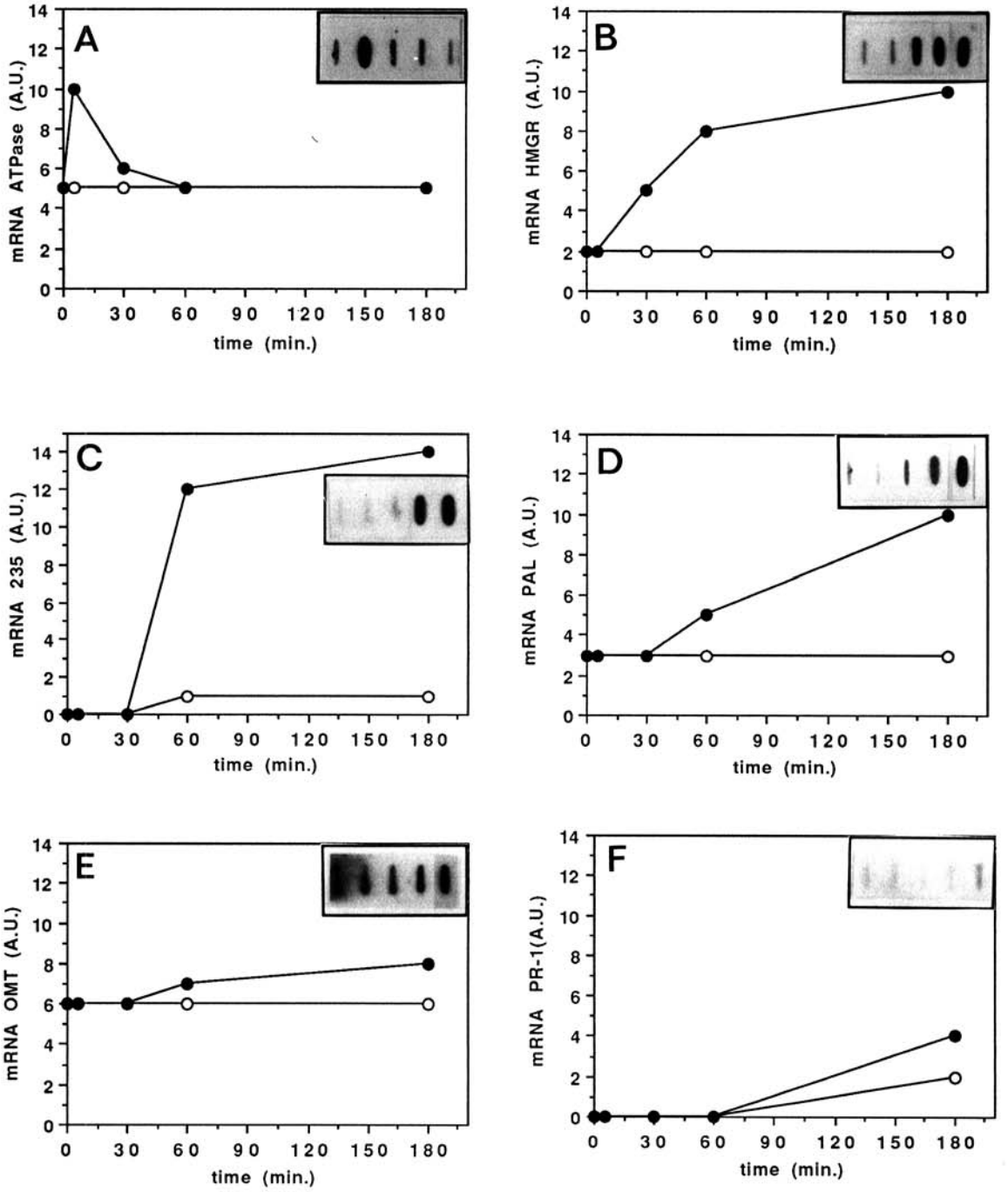

Fig. 5. Accumulation of mRNAs in tobacco cell suspensions treated with 10 nM cryptogein (●) and in untreated cell suspensions (○) (A.U. = arbitrary units): **A,** mRNA encoding plasma membrane H^+-ATPase; **B,** mRNA encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR); **C,** mRNA hybridizable with clone 235; **D,** mRNA encoding phenylalanine ammonia lyase (PAL); **E,** mRNA encoding *O*-methyltransferase (OMT); **F,** mRNA encoding the pathogenesis-related protein PR-b1. Slot blots were performed as described in Materials and Methods, with 200 ng of poly(A) RNA. Probes were labeled by random priming, and mRNA was quantitated by image analysis. The inserts show slot blots obtained with cryptogein-treated cells.

acrylamide/bisacrylamide (24:1 wt/wt), 9.5 M urea, 2% (wt/vol) Nonidet P40, and 3.2% (wt/vol) ampholines 3–10 (Pharmacia), was poured into capillary tubes (0.1 × 8 cm). The resulting pH gradient ranged from 4.7 to 7.3. In each tube, 20 µg of total protein was loaded. The catholyte (upper compartment) consisted of 100 mM NaOH, and the anolyte (lower compartment) consisted of 11 mM H_3PO_4. Isoelectric focusing was run for 15 min at 500 V and then for 2 h at 850 V. Gels were then equilibrated in a modified Görg buffer (Görg et al. 1987): 0.05 M Tris-HCl (pH 8.3) containing 2% sodium dodecyl sulfate (SDS), 10% sucrose, 65 mM dithiothreitol, and 0.002% bromophenol blue. The second dimension was SDS–polyacrylamide gel electrophoresis, performed according to O'Farrell (1975) with the following modifications: the stacking gel was omitted, and running gels contained 12% acrylamide. Electrophoresis was run at 20 mA per gel for 90 min. A Carbamalyte kit (Pharmacia) was used as an external standard. Gels were fixed and stained with silver (Blum et al. 1987). Quantitative measurement of the intensity of silver-stained polypeptide spots was performed with an image analysis system (SAMBA 2005, Alcatel-TITN, Grenoble, France) as described by Oulad Abdelghani et al. (1991). Three independent measurements were performed for each gel, and three gels were analyzed per sample. Standard error was found to vary from 4 to 19%, depending mainly on spot shape. Without knowledge of the specific absorption coefficient of each polypeptide, this method only indicates the relative variations of one given polypeptide: for this reason, the absorbance of each polypeptide spot was expressed as the percentage of the maximal absorbance of the same spot found in all experiments.

RNA preparation.

Total ribonucleic acids were isolated from harvested cells by a phenol-SDS method, and poly(A) RNA was prepared from total RNA by two cycles of affinity chromatography on oligo(dT) cellulose as described by Suty et al. (1993).

In vitro translation and two-dimensional electrophoresis of translated polypeptides.

Total RNA and poly(A) RNA were translated in vitro in the presence of 1.5 mCi of ^{35}S-methionine per milliliter (Amersham) (specific activity greater than 1,000 Ci/mmol) by using a wheat germ extract (Boehringer) according to the manufacturer's recommendations. Labeled polypeptides were precipitated with trichloroacetic acid and dissolved in electrophoresis buffer. Electrophoresis was carried out as described above, and ^{35}S-labeled polypeptides were detected by autoradiography.

Slot blots, Northern blots, and hybridization procedures.

Aliquots of total RNA and poly(A) RNA were denatured in glyoxal (Suty et al. 1993) and layered on Zeta-probe membrane with a slot blot apparatus (Hoefer Scientific). The RNA was fixed for 3 min under UV light (254 nm), and the membranes were heated to 80°C for 1 h in order to reverse the glyoxalation.

For Northern blots total RNA was heat-denatured in a mixture of 2.2 M formaldehyde and 50% formamide for 9 min at 70°C and then electrophoresed in a formaldehyde-denaturing 1% agarose gel. RNAs were transferred to Hybond N membrane (Amersham) by overnight blotting in 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The following cDNA probes were used for hybridizations: the pma2-ATPase cDNA probe from *Nicotiana plumbaginifolia* (Boutry et al. 1989), the HMGR cDNA (clone 6P2) probe from *N. sylvestris* (Genschick et al. 1992), the PR-b1 cDNA probe from *N. tabacum* (Tahiri-Alaoui et al. 1993), and the clone 235 cDNA probe (Marco et al. 1990) corresponding to a gene activated during the interaction of tobacco and *Pseudomonas*. This clone is subject to a confidential agreement and was used only as a positive marker of activation of the HR (Godiard et al. 1991). The probes were ^{32}P-labeled by random priming. Hybridizations were performed at 42°C for 16 h in the following mixture: 50% formamide, 4× SSC, 1% lauryl sarkosyl, 200 µg of denatured salmon sperm DNA per milliliter, 10% dextran sulfate (wt/vol), and 1 ng of labeled probe per milliliter. The filters were washed four times in a mixture of 2× SSC and 0.1% SDS for 5 min at room temperature and once in a mixture of 0.2× SSC and 0.1% SDS for about 15 min at a temperature depending on the specific probe (42 to 65°C).

The filters were then autoradiographed, and hybridizations were quantified by image analysis (Samba 2005). Hybridizations with PAL cDNA (Pellegrini et al. 1994) and tobacco OMT I cDNA were carried out on the same membranes by S. Kauffmann (IBMP, Strasbourg) and quantified by the use of a Phospho-Imager (Molecular Dynamics). Hybridizations with HMGR probe carried out on the same membranes and quantified by the two image analysis systems allowed a comparison of the results.

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