Planta (1992) 187:163–170

Planta

Movement of elicitins, necrosis-inducing proteins secreted by *Phytophthora sp.*, in tobacco

Amélie Zanetti, Françoise Beauvais, Jean-Claude Huet, and Jean-Claude Pernollet*

Laboratoire d'Etude des Protéines, Département de Physiologie et Biochimie Végétales, INRA, Route de St. Cyr, F–78026 Versailles Cedex, France

Received 14 May; accepted 13 September 1991

Abstract. In culture, *Phytophthora* fungi – except *P. nicotianae* – secrete proteins, called elicitins, which cause necrosis on the leaf of the non-host tobacco (*Nicotiana tabacum* L.) at a distance from the inoculation site, and are responsible for the incompatible reaction. Cryptogein and capsicein are elicitins secreted by *P. cryptogea* and *P. capsici*, respectively, and form part of a novel family of 10-kDa holoproteins. On tobacco, the necrotic activity of cryptogein is approx. 100-fold higher than that of capsicein. Using elicitins radioactively labelled in vivo, we have demonstrated that cryptogein and capsicein (i) move from a wound in the stem towards the leaves where they interact directly, (ii) reach their target without undergoing any molecular alteration, (iii) are carried in, and at the same rate as, the sap flow in the xylem, (iv) do not alter the rate of the xylem flow although they are able to provoke drastic damage to the lamina. Consequently, the remote necrotic activity of elicitins does not require any transportable secondary plant elicitor, so the differences in necrotic properties should be due to structural features involved in the interaction of elicitins with the leaf target cells.

Key words: Cell signalling – Elicitor – *Nicotiana* (elicitor movement) – *Phytophthora* – Transport (elicitor)

Introduction

Numerous elicitors of necrosis produced by fungi (Bowen and Heale 1987) or during the plant-fungus interaction (De Wit et al. 1985) have been described and are considered to be responsible for the induction of the hypersensitive reaction, serving as signals for the interaction between plant and pathogens (Dixon and Lamb 1990). Upon inoculation, *Phytophthora nicotianae*, the agent of tobacco black shank, invades tobacco stems, whereas other *Phytophthora* species cause limited colonization accompanied by distant leaf necrosis, i.e. leaf necrosis at a distance from the inoculation site. Csinos and Hendrix (1977, 1978) observed that several *Phytophthora* species were able to release into the culture medium a toxin active on tobacco. In contrast, no distant leaf necrosis was observed during the interaction between *P. nicotianae* and tobacco, and no elicitor of necrosis was detected in culture filtrates (Bonnet 1985). In culture, *P. cryptogea* and *P. capsici* secrete low-relative-molecular-mass (M_r) holoproteins, named cryptogein and capsicein respectively, which have been purified (Huet et al. 1990). These proteins act as fungal signals in the plant-*Phytophthora* interactions and are responsible for the tobacco incompatible reaction as defined by Lamb et al. (1989). When applied to tobacco plants, they elicit systemic remote leaf necrosis, cause the accumulation of pathogenesis-related proteins (Bonnet et al. 1986), and induce protection against a subsequent inoculation with the tobacco pathogen *P. nicotianae* (Ricci et al. 1989). In tobacco cell-suspension cultures, cryptogein has been shown to elicit the production of ethylene and phytoalexin (Blein et al. 1991). Elicitins from various *Phytophthora* species exhibit different levels of biological activity: cryptogein causes visible leaf necrosis when applied at approx. 1 μg per plant whereas 50- to 100-fold as much capsicein is required for the same reaction; however, both elicitins have the same ability to induce protection (Ricci et al. 1989).

The complete amino-acid sequences of cryptogein and capsicein are known (Ricci et al. 1989) each consisting of 98 residues. They are holoproteins devoid of glycosylation, with a sequence homology of 85%. Only two short terminal regions are heterologous, and these regions are probably involved in the modulation of the biological activities (Huet and Pernollet 1989; Nespoulous et al. 1992). As leaf symptoms occur at a distance from the application site and would involve a systemic movement of the elicitin molecule, differences in necrotic activity

* To whom correspondence should be addressed

Abbreviations: M_r = relative molecular mass; RPLC = reverse-phase liquid chromatography; SDS–PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

between cryptogein and capsicein might relate to differences in translocation properties. Although it is indicated by the effect of cryptogein on tobacco cell cultures (Blein et al. 1991), it is not known whether elicitins are primary elicitors acting directly in the leaf lamina. Since the activation of an oligogalacturonide fungal elicitor was observed to occur through a plant enzymatic reaction (Cervone et al. 1989), it may be asked whether cryptogein and capsicein also require in-planta biochemical alterations for their activity to develop. This paper reports for the first time the movement of a secreted fungal protein elicitor inside a plant host towards its remote site of action.

Materials and methods

Elicitin purification. Elicitins were purified using a previously described two-step chromatographic procedure (Huet et al. 1990; Nespoulous et al. 1992). Ion-exchange chromatography on SP–Zeta Prep disks (Flot-Cuno, Boissy-St. Léger, France) and Sephadex G–50 gel filtration (Pharmacia-France, St. Quentin en Yvelines) in pure water were used for cryptogein. Capsicein, which is acidic, was submitted to reverse-phase liquid chromatography (RPLC) in ammonium-acetate buffer before Sephadex G–50 gel filtration in pure water.

Labelling of cryptogein and capsicein with ^{35}S. Phytophthora cryptogea (isolate A52) and *P. capsici* (isolate A147) from the *Phytophthora* collection at INRA Antibes, France, were cultured in Petri dishes in the presence of 185 MBq of ^{35}S-sulphate (Amersham-France, Les Ulis; ref. SJS1, batch 223AC, 30 GBq · μmol^{-1}) in a culture medium adapted from Plich and Rudnicki (1979) in which $MgSO_4 \cdot 7H_2O$ was half replaced by $MgCl_2 \cdot 6H_2O$. The medium was sterilized by filtration on Sterivex-GS 0.22-μm filters (Millipore-France, St. Quentin en Yvelines). After 15 d of culture in darkness at 26° C, culture filtrates were filtered through 0.22-μm μstar filters (Costar; Poly-Labo, Strasbourg, France). Both labelled capsicein and cryptogein were purified directly by RPLC, using a Spectra Physics chromatographic system (Spectra Physics-France, Les Ulis) composed of an 8700 XR LC pump, an 8750 organizer, an 8773 XR UV detector and an ISCO 1570 fraction collector. Elicitins were chromatographed in a CH_3COONH_4 (25 mM, pH 7.2)/ CH_3CN system. All solvents (UV grade) were continuously degassed with helium. The culture filtrates (approx. 150 ml), adjusted to pH 7.0, were loaded using the high-pressure pump on an Aquapore (C_8) RP 300 (Brownlee, Applied Biosystems-France, Roissy) cartridge (30 mm long, 10 mm i.d.) at 2.5 ml · min^{-1}, and eluted at room temperature (22° C). Elution was conducted with a gradient of CH_3CN (FISONS, far-UV grade; Touzart et Matignon, Vitry-sur-Seine, France) obtained using solvents A (5% CH_3CN/ 95% 25 mM CH_3COONH_4 (pH 7.2) and B (50% CH_3CN/50% 50 mM CH_3COONH_4 (pH 7.2). The gradient – from 5 to 18.5% CH_3CN in 10 min, from 18.5 to 36.5% in 20 min, from 36.5 to 50% in 5 min, washing for 5 min at 50% CH_3CN followed by a 15 min re-equilibration at 5% CH_3CN – was applied at a flow rate of 2.5 ml · min^{-1}. A 200-μl aliquot (approx. 1% in volume) of the recovered peak was diluted (1/4, v/v) with water and submitted to analytical RPLC on an Aquapore (C_8) RP300 column (30 mm long, 4.6 mm i.d.) at 0.5 ml · min^{-1}. After 215-nm UV detection, the eluent flow was mixed with a scintillation mixture (Luma Flow II) at a flow rate of 2.5 ml · min^{-1}. The radioactivity was monitored using a Flo-One beta radioactive flow detector (Flotec, La Queuelez-Yvelines, France) with a flow cell of 2.5 ml. Labelled elicitin fractions were lyophilized. In order to obtain a proper specific radioactivity for migration experiments, fractions were diluted with unlabelled elicitins before incorporation in leaves or plants.

Elicitin incorporation and movement in detached leaves. Purified labelled elicitins were tested on expanding detached leaves (approx. 20-cm long) from approx. 60-d-old plants of tobacco (*Nicotiana tabacum* L. cv. Xanthi). Two doses of each labelled elicitin (approx. 1 kBq and 4 kBq, diluted in 10 μl of pure water) were applied to the petiole cut end (approx. 15 mm long) of detached leaves until total incorporation (for 1–2 min) prior to dipping the petioles into a nutrient solution (adapted from Baudet et al. 1986). Unlabelled cryptogein (15 μg) and capsicein (20 μg) were applied as carriers. Elicitin-treated leaves were kept at room temperature in darkness to allow the necrotic symptoms to develop. The detached leaves exhibited nearly constant necrotic symptoms after approx. 16 h. They were then photographed and submitted to autoradiography. Experiments were done in triplicate. The kinetics of movement of ^{35}S-labelled cryptogein and capsicein in detached leaves were followed using a dose of 15 μg elicitin in 3 μl water per leaf (incorporated radioactivity approx. 50 kBq); the drops of elicitin solution were absorbed within 30 to 90 s and the leaves dipped into the nutrient solution from 2 min to several hours. Control experiments were performed using a ^{35}S-labelled sulphate solution (approx. 50 kBq per leaf). Short-term kinetics (2- and 6-min transport times) were obtained by liquid scintillation counting of extract from three parts (proximal, median and distal) of the leaf lamina, cut after autoradiography. The leaf tissues (1 g) were ground by mortar and pestle in 1 ml of 10 mM Tris, 150 mM NaCl buffer (pH 8.0). The extracts were then centrifuged for 10 min at 16 000 · *g* and a 100-μl aliquot solubilized with 500 μl of Lumasolve (Kontron-France, Montigny-le-Bretonneux) and counted in an SL3000 Intertechnique (Kontron-France) counter after addition of 5 ml of Lipoluma scintillator.

Elicitin incorporation and movement in tobacco plants. Movement in whole plants was investigated with ^{35}S-labelled cryptogein and capsicein using a dose of 35–50 μg in 20 μl water per plant (incorporated radioactivity approx. 50–100 kBq). Tobacco plants (cv. Xanthi), cultivated for approx. 60 d in a greenhouse, were decapitated just prior depositing the elicitin solution onto the fresh wound (Ricci et al. 1989). Leaves exhibiting necrotic symptoms were collected after 1 or 2 d (cryptogein and capsicein, respectively). They were then photographed and submitted to autoradiography. After the required exposure time, the leaf necrotic areas were extracted as described below.

Photographs and autoradiographs. Each radioactive leaf (detached or isolated from treated plants) was exposed to Kodak X–OMAT AR film for about 10–30 d at −80° C. The leaves were photographed prior to autoradiography to permit a comparison of the necrotic areas with the location of radioactivity.

Extraction and characterization of elicitins from necrotic lamina. Elicitins were extracted from the leaves of whole plants. Leaf areas exhibiting necrotic symptoms and the presence of radioactivity after autoradiography were cut off, dipped into liquid nitrogen and powdered in a mortar; 1 g of powder was extracted with 1 ml of 10 mM Tris-HCl, 150 mM NaCl buffer (pH 8.0). After centrifugation at 16 000 · *g* for 10 min, the supernatant was submitted to RPLC with a continuous-flow radioactivity determination, under conditions strictly identical to those used for the control of in-vivo labelled elicitins. The RPLC radioactive peaks were submitted to peptide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) in order to determine the M_rs of the elicitins after migration, using standards from the MW–SDS–17 Sigma peptide calibration kit with the proper standard M_r values (Sallantin et al. 1990). Fluorographs were made according to Chamberlain (1979), the film being exposed to the gels for about 7 d at −80° C before development.

Transport of [^{14}C]methyl-glucose. In order to test the influence of elicitin on the sap flow, we applied unlabelled cryptogein and capsicein to detached leaves or intact plants in the same doses as in the previous experiments but in the presence of [^{14}C]methyl-glucose (Amersham; ref. CFA.459, batch B28, approx. 1.92 GBq · mmol^{-1}) at doses of approx. 25 kBq per detached leaf and approx. 50 kBq per decapitated plant. Controls were obtained by replacing the elicitin solutions with pure water.

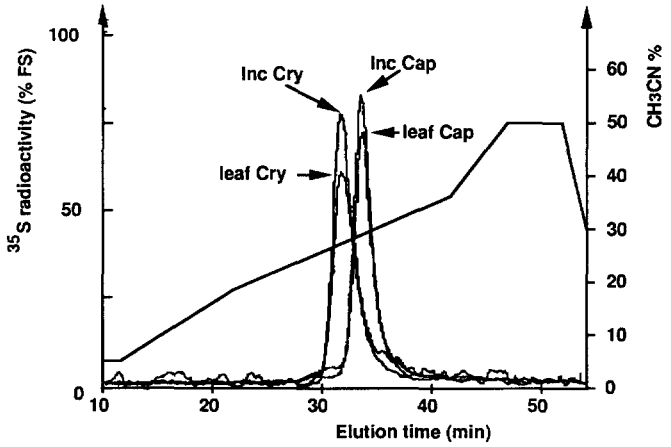

Fig. 1. Comparison of RPLC profiles of ^{35}S-labelled elicitins before and after movement in tobacco plants. *Inc Cry* and *Inc Cap*, ^{35}S-labelled cryptogein and capsicein, respectively, used for incorporation in detached leaves or decapitated plants; *leaf Cry* and *leaf Cap*, cryptogein and capsicein extracted from necrotic areas of the laminae 1 and 2 d after inoculation of decapitated plants, respectively. Radioactivity is expressed as a percentage of full scale (FS = 200–1000 cpm)

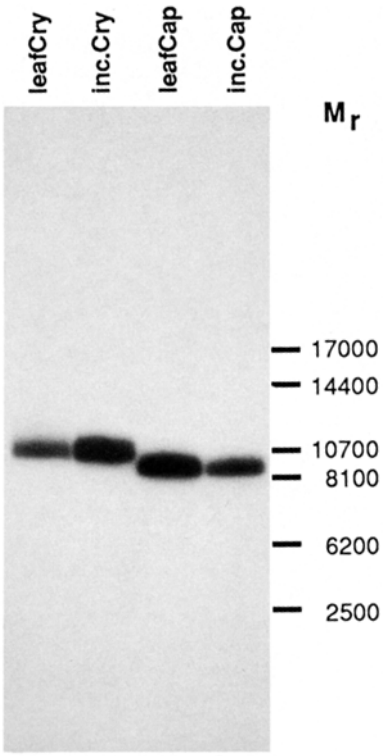

Fig. 2. Comparison of SDS–PAGE profiles of ^{35}S-labelled elicitins before and after movement in tobacco plants. *inc Cry* and *inc Cap*, ^{35}S-labelled cryptogein and capsicein, respectively, used for incorporation in detached leaves or decapitated plants; *leaf Cry* and *leaf Cap*, cryptogein and capsicein extracted from necrotic areas of laminae 1 and 2 d after inoculation of decapitated plants, respectively

Results

Characterization of ^{35}S-labelled elicitins. Figure 1 shows the radioactive RPLC profiles of purified ^{35}S-labelled cryptogein and capsicein. The peaks were single and symmetrical for each elicitin. They eluted respectively at 27.5 and 30.5% CH_3CN, values characteristic of the unlabelled elicitins (Nespoulous et al. 1992). No other radioactive peaks were observed. As shown in Fig. 2, the electrophoretic profiles of these fractions each exhibited only one radioactive band, at the same position as found for the unlabelled elicitin used as carrier and made visible by Coomassie-blue staining (not shown). ^{35}S-labelled elicitins had apparent M_rs identical to those of unlabelled elicitins (Ricci et al. 1989; Sallantin et al. 1990). The specific radioactivity of labelled elicitins varied from 2 to 5 kBq · μg^{-1}, depending on batches. The ^{35}S-radioactivity recovery in secreted elicitins, which have nine sulphur amino acids in their sequence, was around 6% of that initially used in the culture medium. Elicitins accounted for nearly all the labelled proteins and for half of the ^{35}S-radioactivity recovered in the *Phytophthora* culture medium.

Movement of cryptogein and capsicein in detached leaves. Figure 3 allows a comparison of the location of the necrotic areas with the distribution of the radioactive elicitins absorbed by detached leaves, observed on the same leaf for each elicitin. With either cryptogein or capsicein, after 24 h nearly the whole leaf was covered with necroses and the presence of radioactivity was more or less observed everywhere in the lamina although the vascular bundles were not labelled. With the elicitin doses utilized, necroses appeared more quickly with cryptogein (approx. 12 h) than with capsicein (approx. 24 h). However, under our conditions, there was no appreciable spread after 24 h.

Figure 4 shows comparisons of the extents of movement of cryptogein and capsicein in detached tobacco leaves inoculated with a 50-fold higher amount of radioactivity than in the previous experiment in order to increase the sensitivity of detection. Autoradiographs of tobacco leaves inoculated with ^{35}S-labelled cryptogein and capsicein, 2 h after inoculation, were similar to those of Fig. 3 and clearly showed the invasion of the whole lamina by both radioactive elicitins. No change in the autoradiographs could be observed after inoculation-times longer than 2 h. As soon as 10 min after inoculation, radioactive labelling occurred in the leaf parenchyma (Fig. 4). Besides, a weak label caused by cryptogein and capsicein could be detected in the vascular bundles. This label, however, decreased within the next hours (Fig. 4, compare 10 min and 2 h). Labelling by ^{35}S-sulphate, however, resulted in a pronounced trace in the vascular bundles independent from the time the autoradiograms were taken (Fig. 4, CTL). There were no obvious differences in the diffusion rates of cryptogein, capsicein and the sulphate used as a control. In the latter case, in contrast to elicitin-treated leaves, the vascular bundles were also labelled.

The rapid transport of elicitins in detached leaves is demonstrated in Fig. 5B which compares the amount of ^{35}S-radioactivity (expressed as percentage of the total radioactivity applied) associated with cryptogein and capsicein with that of ^{35}S-sulphate found in three areas

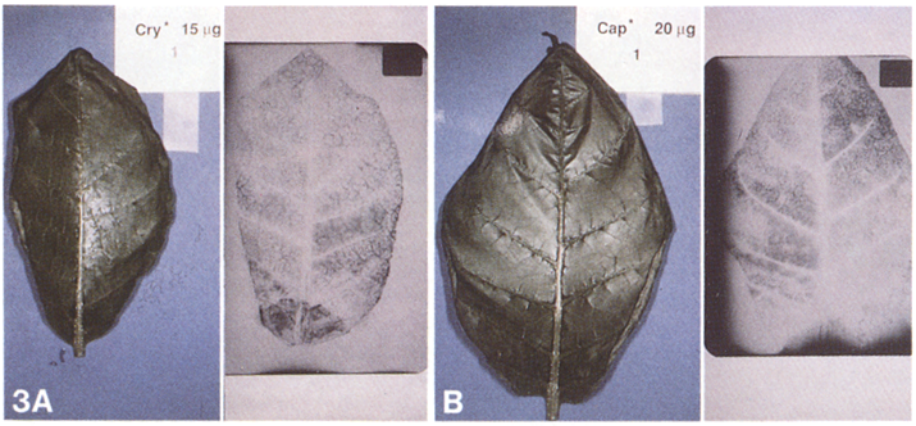

Fig. 3A, B. Movement of cryptogein and capsicein in detached tobacco leaves: comparison of the location of necrotic symptoms with that of ^{35}S radioactivity. Photographs *(left)* compared with autoradiographs *(right)* of detached tobacco leaves inoculated with **A** 1400–4300 Bq ^{35}S-labelled cryptogein (15 µg per leaf); **B** 960–2900 Bq ^{35}S-labelled capsicein (20 µg per leaf). Leaves were collected approx. 1 d after inoculation

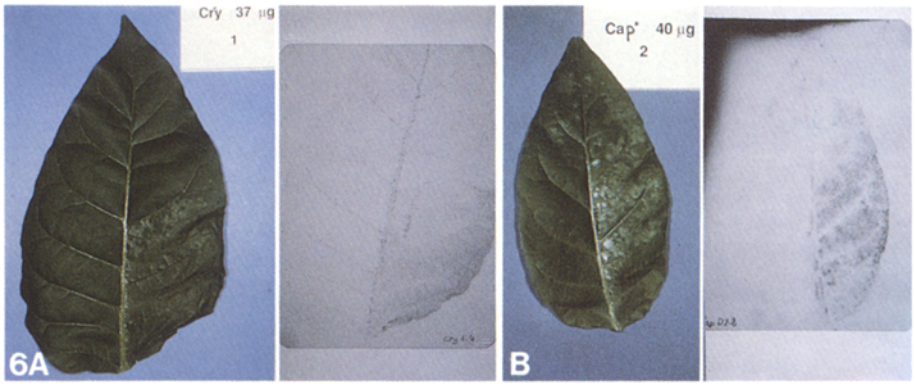

Fig. 6A, B. Movement of cryptogein and capsicein in tobacco plants: comparison of the distribution of necrotic symptoms with that of ^{35}S radioactivity. Photographs *(left)* and corresponding autoradiographs *(right)* of tobacco leaves excised from decapitated plants inoculated with: **A** 56 kBq ^{35}S-labelled cryptogein (37 µg per plant); **B** 107 kBq ^{35}S-labelled capsicein (40 µg per plant). Leaves were collected approx. 1 and 2 d after inoculation, for cryptogein and capsicein, respectively

of leaf after 2 and 6 min of migration. Two minutes after application of elicitin solution, elicitins were present in the proximal part of the leaf. Between 2 and 6 min, the labelled elicitins had progressed towards the median and distal parts, but a major proportion of the radioactivity (approx. 65–80%) was still in the petiole. No appreciable difference was observed between cryptogein and capsicein which both moved at approximately the same rate as ^{35}S-sulphate. The mean elicitin rate of movement in our conditions was approx. 1–3 cm · min^{-1}. The relative proportion of radioactivity in the proximal part after 6 min in a leaf inoculated with cryptogein was found to be slightly lower than that in the median part, while a large fraction of radioactivity was still in the petiole. This could be explained by the size of the proximal part which was cut relatively smaller than the median part, and by a possible diffusion of cryptogein backwards to the nutrient solution.

Movement of cryptogein and capsicein in tobacco plants. When elicitins were applied to decapitated plants, necrotic symptoms appeared in several leaves and their distribution was generally dependent on leaf position occurring preferably and more rapidly in younger leaves. No difference was observed in the distribution of cryptogein and capsicein among the leaves, except that necroses were more severe and occurred more quickly when cryptogein was used, stabilizing after 1 d instead of 2 d with capsicein. In contrast to detached leaves, only a part of the lamina became necrotic, the necroses occurring initially as spots. Figure 6 shows two leaves with these partial necroses: in the case of the cryptogein-treated plant, the necrotic spots were mainly distributed on the right-hand proximal part of the leaf (Fig. 6A) whereas in the capsicein-treated plant, necrotic spots were observed on the whole right-hand side of the leaf (Fig. 6B) as well as some at the left distal end. In both cases the necrotic areas were correlated with the presence of radioactivity.

Identification of the radioactive compounds isolated from the leaf necrotic areas as unaltered elicitin molecules. Figure 1 shows comparisons of the radioactive RPLC profiles of purified in-vivo ^{35}S-labelled elicitins before incorporation into decapitated tobacco plants with those extracted from the necrotic leaf tissue 1 or 2 d after inoculation. In both cases, only one radioactive peak for each elicitin was observed during the whole chromatographic process, from injection to column washing. When chromatographed under exactly the same conditions, cryptogein and capsicein eluted respectively at approx. 27.5% and 30.5% CH_3CN, both before and after migration in the leaf tissue. No ^{35}S-labelled peptides corresponding to cleaved fragments could be observed by this technique. Peptide SDS–PAGE of elicitins extracted from necrotized leaves, shown in Fig. 2, demonstrated that the M_rs of elicitins extracted after movement in the whole plant towards the necrotized lamina were strictly identical to those of the native labelled proteins, and that no degradation of the molecules could be observed. The radioactivity detected in the necrotized parts of the leaves was therefore due to authentic unaltered elicitins.

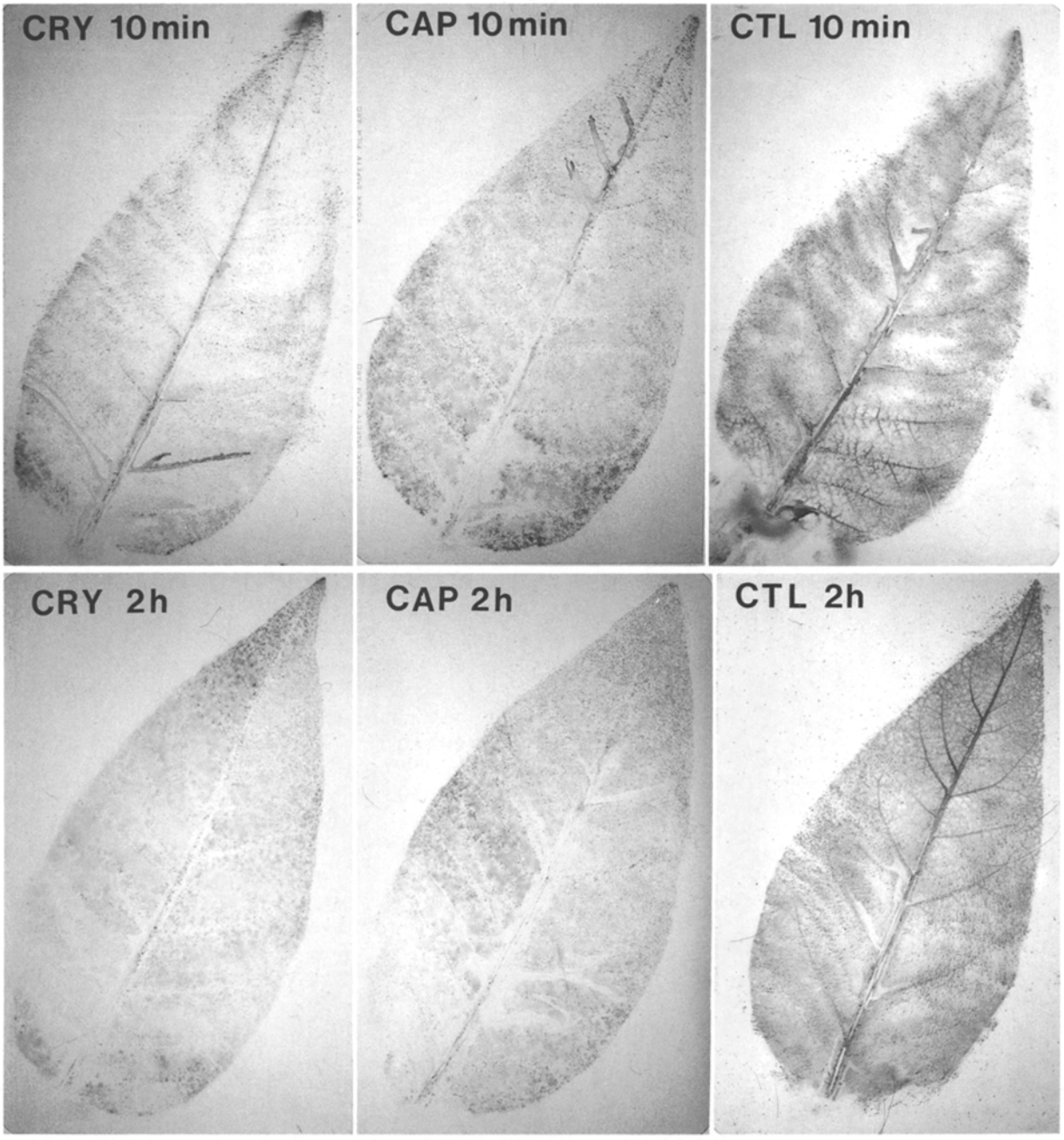

Fig. 4. Kinetics of movement of elicitins in detached tobacco leaves. Autoradiographs of tobacco leaves inoculated with ^{35}S-labelled cryptogein (*CRY;* 15 µg, 27 kBq per leaf), capsicein (*CAP;* 15 µg, 30 kBq per leaf) and control *(CTL)* leaves inoculated with ^{35}S-labelled sulphate (45 kBq per leaf), 10 min and 2 h after inoculation

Influence of elicitins on the sap flow in the xylem of the leaves. Since elicitins altered the plant leaf tissues, we asked whether they were able to modify the transport of sap by damaging the vascular elements, thereby weakening certain leaf areas and rendering them more susceptible to elicitin. Figure 7 shows the results of experiments on the migration of [^{14}C]methyl-glucose, a glucose analogue unable to enter the metabolic pathways, in the presence or absence of unlabelled elicitins. The detached leaves treated with cryptogein or capsicein were entirely necrotized (not shown), in contrast to the untreated control leaf (not shown), but no differences in the partitioning of [^{14}C]methyl-glucose radioactivity were observed (Fig. 7A). A similar result was obtained with decapitated plants (Fig. 7B), even if excised leaves were only partially necrotized (proximal part for cryptogein experiment, left proximal part for capsicein-treated tobacco plant). As observed with ^{35}S-sulphate and in contrast to elicitins, [^{14}C]methyl-glucose was detected in the vascular bundles. In no case was the transport of the [^{14}C]methyl-glucose tracer modified by cryptogein or capsicein, even when necroses had widely developed in the lamina.

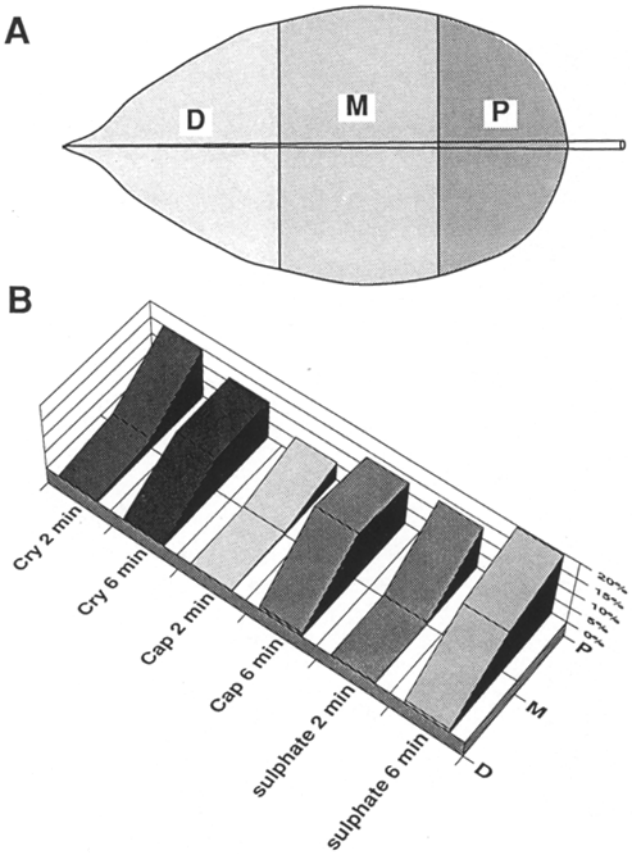

Fig. 5A, B. Short-term kinetics (2 and 6 min) of movement of elicitins in detached tobacco leaves. **A** Diagram of leaf showing positions of cuts and areas used for assay; *P*, proximal area; *M*, median part. *D*, distal part; **B** Histogram showing percentage of total incorporated radioactivity recovered in each part of the leaf. *Cry*, ^{35}S-labelled cryptogein (15 µg, 27 kBq per leaf); *Cap*, capsicein (15 µg, 30 kBq per leaf); sulphate, ^{35}S-labelled sulphate (45 kBq per leaf)

Discussion

Both experiments with detached leaves and whole plants showed that elicitins migrated from the inoculation site to the necrotic leaf areas. Moreover, the radioactivity found in the leaves was undoubtedly solely based on the presence of native unaltered elicitins. Even after 2 d of transport from the top of the plant to the necrotic areas of the leaves there was no alteration in the size or hydrophobicity of the elicitins, and no elicitin fragments could be observed. The spatial correlation between necroses and radioactivity demonstrated that the elicitins were directly the primary cause of the severe tissue damage. The ^{35}S-labelling was chosen because in the elicitin molecules (Huet and Pernollet 1989) nine residues out of 98 are sulphur amino acids regularly distributed from the N-terminus to the C-terminus (the first cysteine occurs at position 3 and the last at position 95). Any cleavage would have resulted in the release of ^{35}S-labelled peptides which would have been detected either by RPLC or by peptide SDS–PAGE. In contrast to the observed activation of an oligogalacturonide fungal elicitor by a plant enzyme (Cervone et al. 1989), cryptogein and capsicein do not require any in-planta alteration. The integrity of elicitins after transport is in accordance with the observation that all of their cysteine residues are involved in disulfide bonds (Nespoulous et al. 1992); such bonds occur at each end of the molecule, preventing elicitins from enzymatic cleavage by exopeptidases.

In detached leaves the rate of elicitin migration was the same as that of ^{35}S-sulphate (approx. 1–3 cm · min^{-1}). Elicitins were probably carried passively by the sap flow in the xylem. A weak labelling by ^{35}S-elicitins could be detected in the vascular bundles after short-term inoculation (Fig. 4). This labelling decreased with increasing time of elicitin-treatment and spread over the lamina. In contrast to sulphate, the elicitins accumulated in the lamina of the leaves. They did not alter the sap flow as demonstrated with the tracer [^{14}C]methyl-glucose even though this moved more slowly than sulphate. The comparison of the translocation rates of the elicitins with the development of necroses indicates that 4–8 h are necessary for the cell damage to become observable, once the lamina cells had been reached by the elicitin molecules. This may be compared with the 2- to 4-h delay for tobacco cell-suspension cultures to produce ethylene or capsidiol (Blein et al. 1991). The asymmetry of elicitin migration and the partitioning of the necrotized leaves observed in decapitated-plant experiments may be an artifact resulting from the blockage of the vessels by air bubbles (Sperry and Tyree 1988; Tyree and Sperry 1989). In experiments on detached leaves, the initial appearance of necrotic spots and their spread throughout the whole lamina were rapid, in contrast to decapitated plants in which symptoms took longer to develop. The dilution of elicitin in the whole plant, rather than a delay in transport, would explain such differences.

Whereas the production of phytoalexins in soybean and parsley has been shown to be elicited by different cell-wall components from *P. megasperma* (Parker et al. 1988), elicitins, proteins secreted by numerous *Phytophthora* species, appear to play an active role in the plant attack. They were indeed actively secreted by both *P. cryptogea* and *P. capsici* since 6% of the total available ^{35}S-radioactivity was found in elicitin protein; this accounted for half of the radioactivity recovered in the culture medium. We also showed that the necrosis-inducing activity of the elicitins did not require any migrating secondary plant elicitor. As far as we know, elicitins are the first known holoproteins to be shown to undergo long-distance translocation inside a plant. Although a glycoprotein produced by *P. parasitica* var. *nicotianæ* (Farmer and Helgeson 1987) has already been found within tobacco plants, its molecular integrity and the possible correspondence between its location and activity was not investigated.

Since cryptogein and capsicein were able to reach the target cells and did not require any transportable secondary plant elicitor, the cryptogein activities which were observed in tobacco cell suspension cultures (Blein et al. 1991) were probably the same as those occurring in whole tobacco plants. The origin of the difference in necrotic properties of α and β elicitins (Nespoulous et al. 1992) is likely to be found in structural features involved in the

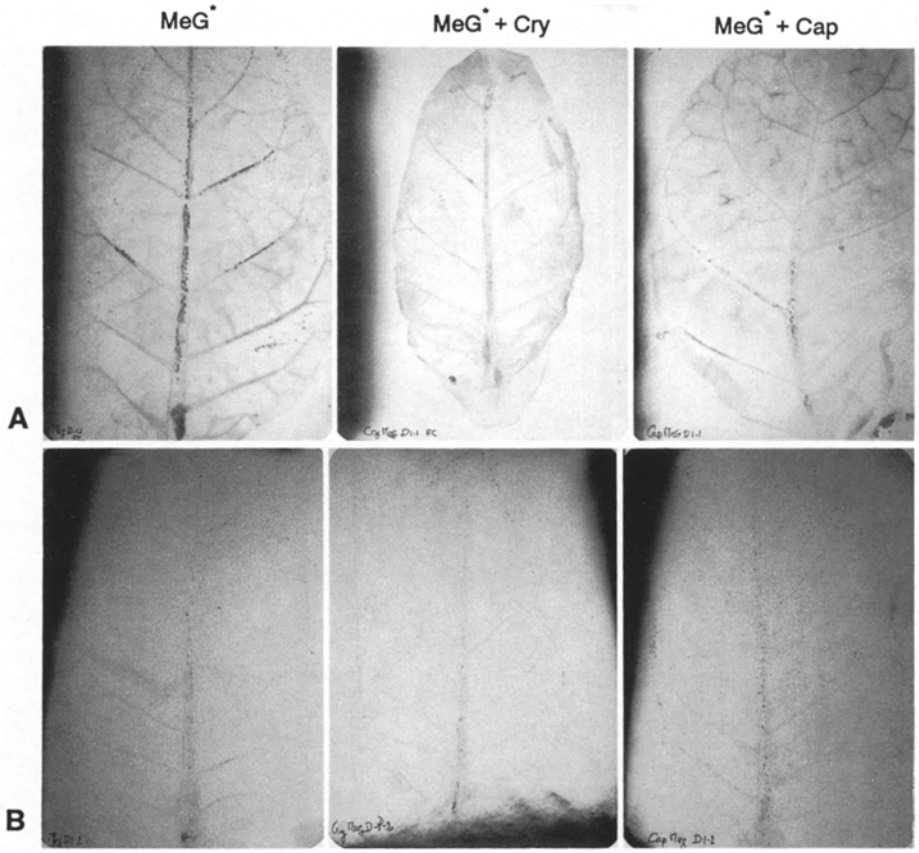

Fig. 7A, B. Influence of elicitins on sap transport in vessels in detached leaves and decapitated tobacco plants. Autoradiographs of tobacco leaves after migration of [^{14}C]methyl-glucose applied in the presence or absence of unlabelled elicitins. **A** Detached leaves observed 1 d after elicitin inoculation; *MeG**, [^{14}C]methyl-glucose solution (5.5 kBq per leaf); *MeG* + Cry*, [^{14}C]methyl-glucose solution (5.5 kBq per leaf) plus unlabelled cryptogein (15 µg per leaf); *MeG* + Cap*, [^{14}C]methyl-glucose solution (5.5 kBq per leaf) plus unlabelled capsicein (20 µg per leaf). **B** Leaves excised from decapitated tobacco plants collected approx. 1 and 2 d after inoculation, for cryptogein and capsicein, respectively; *MeG**, [^{14}C]methyl-glucose solution (11 kBq per plant); *MeG* + Cry*, [^{14}C]methyl-glucose solution (11 kBq per plant) plus unlabelled cryptogein (30 µg per plant); *MeG* + Cap*, [^{14}C]methyl-glucose solution (11 kBq per plant) plus unlabelled capsicein (40 µg per plant)

interaction of elicitins with the target cells in the leaf lamina. The biological properties described in this paper supplement the structural data already obtained concerning elicitins, and support the hypothesis that elicitins not only act as toxins but are signalling molecules which induce the plant defense mechanisms.

The authors are indebted to Mauricette Sallé-Tourné, Marc Sallantin and Christian Ouali for their skilful technical assistance.

References

Baudet, J., Huet, J.C., Jolivet, E., Lesaint, C., Mossé, J., Pernollet, J.C. (1986) Changes in accumulation of seed nitrogen compounds in maize under conditions of sulphur deficiency. Physiol. Plant. **68,** 608–614

Blein, J.P., Milat, M.L., Ricci, P. (1991) Responses of cultured tobacco cells to cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*. Plant Physiol. **95,** 486–491

Bonnet, P. (1985) Réactions différentielles du tabac à 9 espèces de *Phytophthora*. Agronomie **5,** 801–808

Bonnet, P., Poupet, A., Abad, P., Venard, P., Cardin, L. (1986) Induction de nécroses foliaires, de protéines b et de résistance dans les interactions tabac – *Phytophthora*. Agronomie **6,** 829–837

Bowen, R.M., Heale, J.B. (1987) Four "necrosis-inducing" components, separated from germination fluid of *Botrytis cinerea* induce active resistance in carrot root tissue. Physiol. Mol. Plant Pathol. **30,** 55–66

Cervone, F., Hahn, M.G., De Lorenzo, G., Darvill, A., Albersheim, P. (1989) Host-Pathogen interactions. XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. **90,** 542–548

Chamberlain, J.P. (1979) Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. **98,** 132–135

Csinos, A., Hendrix, J.W. (1977a) Nonparasitic stunting of tobacco plants by *Phytophthora cryptogea*. Can. J. Bot. **55,** 26–29

Csinos, A., Hendrix, J.W. (1977b) Toxin produced by *Phytophthora cryptogea* active on excised tobacco leaves. Can. J. Bot. **55,** 1156–1162

Csinos, A., Hendrix, J.W. (1978) *Phytophthora* species producing toxin active on tobacco. Soil Biol. Biochem. **10,** 47–51

De Wit, P.G.J.M., Hofman, A.E., Velthuis, G.C.M., Kuc, J.A. (1985) Isolation and characterization of an elicitor of necrosis isolated from intercellular fluids of compatible interactions of *Cladosporium fulvum* (syn. *Fulvia fulva*) and tomato. Plant Physiol. **77,** 642–647

Dixon, R.A., Lamb, C.J. (1990) Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41,** 339–367

Farmer, E.E., Helgeson, J.P. (1987) An extracellular protein from *Phytophthora parasitica* var. *nicotianæ* is associated with stress metabolite accumulation in tobacco callus. Plant Physiol. **85,** 733–740

Huet, J.C., Pernollet, J.C. (1989) Amino acid sequence of cinnamomin, a new member of the elicitin family, and its comparison to cryptogein and capsicein. FEBS Lett. **257,** 302–306

Huet, J.C., Sallantin, M., Pernollet, J.C. (1990) Large scale purification of elicitins, protein elicitors secreted by the plant pathogenic fungi *Phytophthora*. In: Technologies de purification des protéines, vol. 4, pp. 29–34, Briand, Y., Doinel, C., Faure, A., eds, G.R.B.P., Villebon/Yvette, France

Lamb, C.J., Lawton, M.A., Dron, M., Dixon, R.A. (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56,** 215–224

Nespoulous, C., Huet, J.C., Pernollet, J.C. (1992) Structure-function relationships of α and β elicitins, signal proteins involved in the plant-*Phytophthora* interaction. Planta, **186,** 551–557

Parker, J.E., Hahlbrock, K., Scheel, D. (1988) Different cell-wall components from *Phytophthora megasperma* f. sp. *glycinea* elicit phytoalexin production in soybean and parsley. Planta **176,** 75–82

Plich, M., Rudnicki, R.M. (1979) Studies of the toxins of *Phytophthora cactorum* pathogenic to apple trees. I. Isolation, some of the properties and activities of a toxin produced by the fungus cultured in vitro. Phytopath. Z. **94,** 270–278

Ricci, P., Bonnet, P., Huet, J.C., Sallantin, M., Beauvais-Cante, F., Bruneteau, M., Billard, V., Michel, G., Pernollet, J.C. (1989) Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco. Eur. J. Biochem. **183,** 555–563

Sallantin, M., Huet, J.C., Demarteau, C., Pernollet, J.C. (1990) Reassessment of commercially available molecular weight standards for peptide sodium dodecyl sulfate-polyacrylamide gel electrophoresis using electroblotting and microsequencing. Electrophoresis **11,** 34–36

Sperry, J.S., Tyree, M.T. (1988) Mechanism of water stress-induced xylem embolism. Plant Physiol. **88,** 581–587

Tyree, M.T., Sperry, J.S. (1989) Vulnerability of xylem to cavitation and embolism. Annu. Rev. Plant Physiol. Mol. Biol. **40,** 19–38