Research Note

Amino Acid Sequence and Toxicity of the α Elicitin Secreted with Ubiquitin by *Phytophthora infestans*

Jean-Claude Huet, Mauricette Sallé-Tourne, and Jean-Claude Pernollet

Laboratoire d'Etude des Proteines, Département de Physiologie et Biochimie végétales, INRA, Versailles, Route de St Cyr, F-78026 Versailles Cedex, France

Received 28 October 1993. Accepted 28 December 1993.

A single α elicitin, phytotoxin secreted by *Phytophthora infestans*, responsible for systemic HR-like necroses in tobacco, was purified from the culture filtrate. Its sequence was compared to other α elicitins in correlation with toxicity. In the filtrate, we also found ubiquitin, whose biological function remains unclear.

Additional keywords: protein elicitor; *Nicotianae tabacum*; toxin.

Phytophthora infestans was reported to produce toxin(s) in culture, causing necroses in potato, inducing phytoalexin accumulation (Behnke and Lönnendonker 1977; Rönnebeck 1956; Stolle and Schöber 1984, 1985a, 1985b), and killing protoplasts (Möllers *et al.* 1992). Although they were suggested to be macromolecules (Stolle and Schöber 1984; Stolle and Schöber 1985b), which suggests they could consist of elicitins, their chemical nature is still unknown. Except for *Phytophthora parasitica* var. *nicotianae*, all the *Phytophthora* species so far studied secrete elicitins in culture (Pernollet *et al.* 1993), 98-residue holoproteins (Nespoulous *et al.* 1992) that proceed to the leaf they directly necrotize (Zanetti *et al.* 1992). They can induce systemic resistance against pathogens, not only *Phytophthora* fungi (Ricci *et al.* 1989), but also bacteria (Kamoun *et al.* 1993). Various elicitins exhibit different levels of toxicity but induce protection at the same dose (Ricci *et al.* 1989). They are toxic to several plants, in particular potato (Kamoun *et al.* 1993; Pernollet *et al.* 1993). Sequences and local structural differences lead to classifying them in α class (acidic molecules with a valine at position 13) and β class (basic with a hydrophilic residue 13), β elicitins being much more toxic to tobacco (Huet and Pernollet 1989, 1993; Huet *et al.* 1992, 1993; Nespoulous *et al.* 1992; Nespoulous and Pernollet 1993; Pernollet *et al.* 1993).

P. infestans (isolate 90-20-3, race 1,3,4,10,11 from the fungi library of Ploudaniel INRA station) was grown during 3 wk (Pernollet *et al.* 1993). The crude filtrate high-performance liquid chromatography (HPLC) profile was more intricate than were those of other *Phytophthora* species (Huet *et al.* 1992; Huet and Pernollet 1993; Pernollet *et al.* 1993). The major peak, eluting at 30% CH_3CN, showed an α elicitin N-terminal sequence. When the culture filtrate of *P. infestans* was directly submitted to sulfopropyl ion-exchange chromatography, the retained fraction, rechromatographed on Sephadex G50, revealed three peaks (Fig. 1). The peak 2 N-terminus (M Q I F V K T L T G K T I T L D V E P S) was rigorously identical to that of the ubiquitin, whose gene was described by Pieterse *et al.* (1991) in the *P. infestans* genome. We showed that ubiquitin was secreted into the medium during *in vitro* culture. Its biological role in the filtrate is somewhat puzzling, because ubiquitin is usually described to function inside of the cell. It might be therefore a component of the unpurified toxin(s) previously described. Ubiquitin was only observed in *P. infestans,* but its presence cannot be excluded in other *Phytophthora* species, because it was not searched in other species.

The elicitin (called infestin) was purified according to the general procedure used for α elicitins (Pernollet *et al.* 1993). Analytical HPLC, denaturing gel electrophoresis, isoelectric focusing, sequencing, and mass spectrometry revealed only one α elicitin isoform and no β one. Because of lower mycelium growth, *P. infestans* only secreted one-third of the elicitin usually observed (Pernollet *et al.* 1993). *P. infestans* elicitin was reduced and alkylated with 4-vinyl-pyridine (Henschen 1986), prior CNBr digestion. After performic acid oxidation, it was also digested with modified trypsin according to Huet *et al.* (1993). The resulting peptides were separated as already described (Huet *et al.* 1992). Automated Edman degradation was performed using an Applied Biosystems 475A sequencer with reagents and methods of the manufacturer. The N-terminus was sequenced up to Ala 38 (34% initial sequencing yield, 93% repetitive yield). The alignment of the peptides allowed the determination of a 98-residue sequence (Fig. 2). Assuming three disulphide bridges, the M_r of *P. infestans* elicitin was calculated with the average isotopic composition to be 10,325.7. It was also determined on a Trio 2000 mass spectrometer (electrospray ion source and quadrupole mass analyzer VG Biotech Manchester, UK) to be 10,325 ± 4. The perfect agreement between these values shows that the *P. infestans* elicitin is deprived of any side chain posttranslational modification. This M_r value is comparable to the other elicitins that vary from 10,161 to 10,373 Da. *P. in-*

Correspondence to: Jean-Claude Pernollet.

MPMI Vol. 7, No. 2, 1994, pp. 302-304
©1994 The American Phytopathological Society

festans elicitin exhibited a calculated acidic pI (4.7) less acidic than the measured one (3.9 ± 0.1). The amino acid composition, determined with an Applied Biosystems 420 H device, was in agreement with the sequence. Like the other elicitins, infestin exhibited 10 Leu, 3 Ile, 6 Cys, and 3 Met and lacked Trp, His, and Arg. Leu, Ser, Thr, and Ala accounted for half of the residues (49 residues). None of the data revealed any microheterogeneity of this α elicitin, the only isoform found in *P. infestans*.

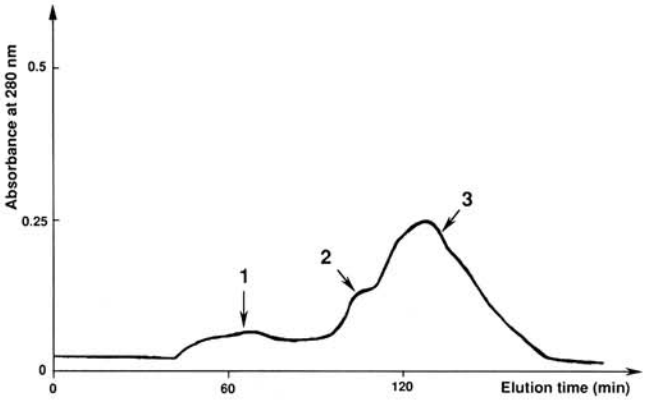

Fig. 1. Preparative exclusion-diffusion chromatography profile of the sulfopropyl ion-exchange chromatography eluate of *Phytophthora infestans* crude filtrate. The chromatography was performed on Sephadex G50 fine (32- × 450-mm column) in Milli-Q water at 3 ml min^{-1}. The three peaks were characterized by N-terminal sequencing: 1, heterogeneous protein content; 2, ubiquitin; 3, salts.

Infestin was comparatively tested with other elicitins on detached tobacco leaves (Nespoulous *et al.* 1992) as shown in Figure 3. The relative necrotic index (ni, defined as the sum of the normalized necrotic area, equal to 1 when a leaf was totally necrotized and 0 for no necrosis, obtained with doses of 100 ng, 1 and 10 μg) was 0.75 for *P. cactorum* elicitin and approximately 1 for parasiticein, capsicein, and cryptogein. The toxicity of *P. infestans* elicitin was found to be higher (ni = 1.4) than of *P. megasperma* var. *megasperma* (ni = 1.1) and *P. drechsleri* (ni = 1.2) α isoforms, but nevertheless less toxic than β cryptogein (ni = 2). Infestin toxicity was also assayed to potato leaves (cv. Roseval). Like capsicein and parasiticein, it was found to be highly necrotic at doses around 1 μg.

The *P. infestans* elicitin sequence was homologous to the 10 other known elicitins, without deletion. With a valine at position 13, it parallels the general classification (Nespoulous *et al.* 1992). Figure 2 shows the comparison of six α elicitins with a percent match of 76.5%. The *P. drechsleri* elicitin was the most different (17 mutations), whereas parasiticein was the closest to infestin (five mutations at positions 6, 10, 68, 85, and 86). The replacement of Pro68 in parasiticein by an alanine in infestin might be the major cause for difference in toxicity. The infestin sequence confirms that the mutations Ala1, Ala10, Thr48, Gly52, and Ser96 are correlated with lower toxicity, but dismisses Ser6 (Huet *et al.* 1993). Together with the investigation of the 3D-structure, in progress through X-ray crystallography (Guilloteau *et al.* 1993) and nuclear magnetic resonance (Bouaziz *et al.*, in press), this information will allow the design of elicitinlike molecules able to elicit the plant defense elicitation but without necrotic properties.

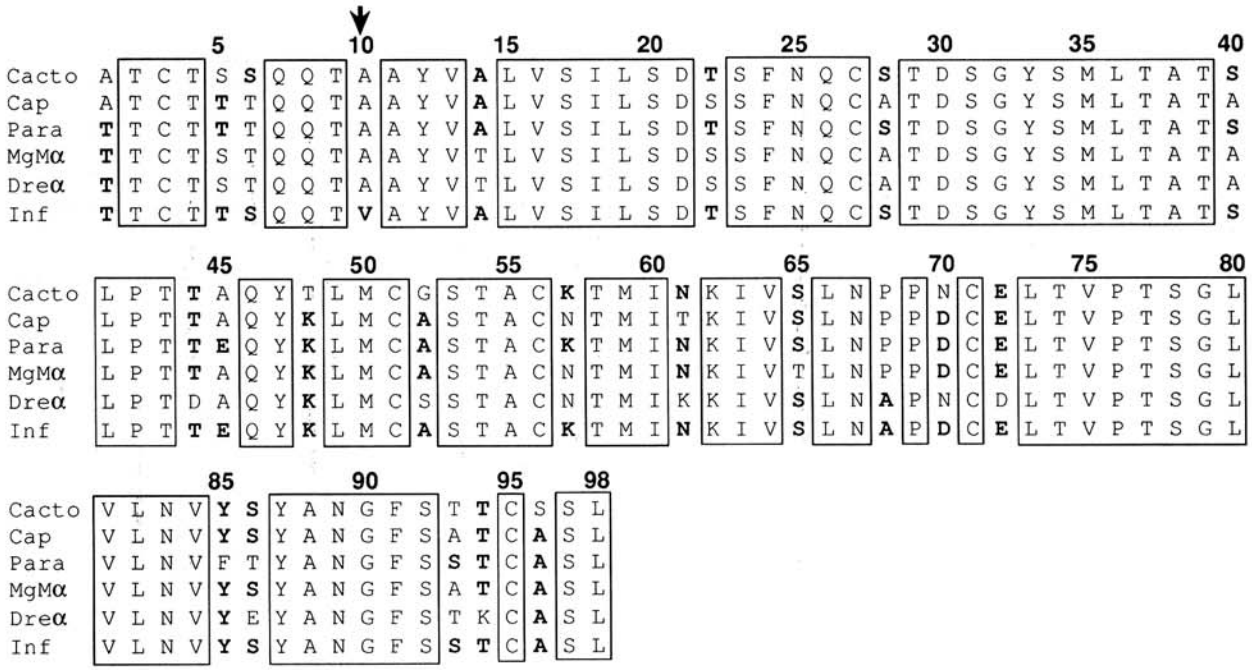

Fig. 2. Comparison of the sequence of the *Phytophthora infestans* elicitin to other α elicitins. Cacto, *P. cactorum* elicitin; Cap, capsicein, *P. capsici* elicitin; Dreα, *P. drechsleri* α elicitin; Inf, *P. infestans* elicitin; MgMα, *P. megasperma* var. *megasperma* α elicitin; Para, parasiticein, *P. parasitica* var. *parasitica* elicitin. The sequences are ordered from top to bottom following increasing elicitin toxicity to tobacco detached leaves. *P. infestans* elicitin was used as a reference; the boxes show the conserved consensus regions; in the variable regions, bold characters emphasize residues identical to those of *P. infestans* elicitin. The arrow indicates the only residue typical of *P. infestans* elicitin.

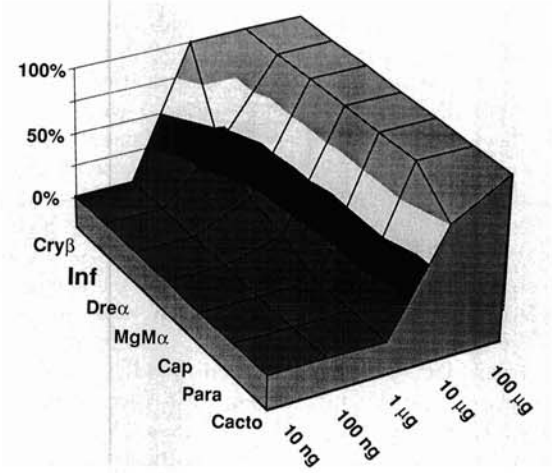

Fig. 3. Necrosis induction on tobacco detached leaves by *Phytophthora infestans* elicitin and other elicitins. Cacto, *P. cactorum* elicitin; Cap, capsicein, *P. capsici* elicitin; Cryβ, β cryptogein, *P. cryptogea* β elicitin; Dreα, *P. drechsleri* α elicitin; Inf, *P. infestans* elicitin; MgMα, *P. megasperma* var. *megasperma* α elicitin; Para, parasiticein, *P. parasitica* var. *parasitica* elicitin. Isoforms are arranged from top to bottom in order of their decreasing toxicity to tobacco. The vertical ordinate indicates the proportion of necrotized area measured on four detached leaves. Elicitins were tested on 60-day-old tobacco plants (cv. Xanthi) cultivated in a greenhouse. Symptoms reached their maximum extent after 2 days. Elicitins were diluted in 10 µl of pure water before being applied to detached leaves.

ACKNOWLEDGMENTS

We would like to warmly thank Jean-Pierre Le Caer (Institut Alfred Fessard, CNRS, Gif-sur-Yvette) for access to mass spectrometry. We are also grateful to Claude Nespoulous for his kind help in writing this manuscript, and to Monique Mansion, Christian Ouali, and Marc Sallantin for their skillful technical assistance.

LITERATURE CITED

Behnke, M., and Lönnendonker, N. 1977. Isolierung und partielle Charakterisierung phytotoxischer Substanzen aus Kulturfiltraten des Pilzes *Phytophthora infestans*. Z. Pflanzenphysiol. 85:17-27.

Bouaziz, S., van Heijenoort, C., Guittet, E., Huet, J. C., and Pernollet, J. C. Resonance assignment, disulfide bridges elucidation and secondary structure determination of capsicein, an α elicitin, by three-dimensional ^{1}H NMR. Eur. J. Biochem. In press.

Guilloteau, J. P., Nespoulous, C., Beauvais, F., Huet, J. C., Pernollet, J. C., and Brunie, S. 1993. Crystallization of β-cryptogein, a toxic elicitor secreted by the phytopathogenic fungus *Phytophthora cryptogea*. J. Mol. Biol. 229:564-565.

Henschen, A. 1986. Analysis of cyst(e)ine residues, disulfide bridges, and sulfhydryl groups in proteins. Pages 244-255 in: Advanced Methods in Protein Microsequence Analysis. B. Wittmann-Liebold, J. Salnikow, and V. A. Erdmann, eds. Springer-Verlag, Berlin.

Huet, J. C., and Pemollet, 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., and Pernollet, J. C. 1993. Sequences of acidic and basic elicitin isoforms secreted by *Phytophthora megasperma megasperma*. Phytochemistry 33:797-805.

Huet, J. C., Mansion, M., and Pernollet, J. C. 1993. Amino acid sequence of the α elicitin secreted by *Phytophthora cactorum*. Phytochemistry 34:1261-1264.

Huet, J. C., Nespoulous, C., and Pernollet, J. C. 1992. Structures of elicitin isoforrns secreted by *Phytophthora drechsleri*. Phytochemistry 31:1471-1476.

Kamoun, S., Young, M., Glascock, C. B., and Tyler, B. M. 1993. Extracellular protein elicitors from *Phytophthora*: Host-specificity and induction of resistance to bacterial and fungal phytopathogens. Mol. Plant-Microbe Interact. 6:15-25.

Möllers, C., Zitzlsperger J., and Wenzel, G. 1992. The effects of a toxin preparation from *Phytophthora infestans* on potato protoplasts and microspores. Physiol. Mol. Plant Pathol. 41:427-435.

Nespoulous, C., and Pernollet, J. C. 1994. Local structural differences between α and β elicitins shown by CD and difference spectroscopy. Int. J. Pept. Prot. Res. 43:154-159.

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

Pernollet, J. C., Sallantin, M., Sallé-Tourne, M., and Huet, J. C. 1993. Elicitin isoforms from seven *Phytophthora* species: Comparison of their physico-chemical and toxic properties to tobacco and other plant species. Physiol. Mol. Plant Pathol. 42:53-67.

Pieterse, C. M. J., Risseeuw, E. P., and Davidse, L. C. 1991. An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. Plant Mol. Biol. 17:799-811.

Ricci, P., Bonnet, P., Huet, J. C., Sallantin, M., Beauvais-Cante, F., Bruneteau, M., Billard, G., and 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.

Rönnebeck, W. 1956. Ein phytotoxisches Prinzip aus *Phytophthora infestans* de By. Z. Planzenkr. Pflanzensch. 63:385-389.

Stolle, K., and Schöber, B. 1984. Wirkung eines Toxins von *Phytophthora infestans* (Mont.) de Bary auf Kartoffelknollengewebe. Potato Res. 27:173-184.

Stolle, K., and Schöber, B. 1985a. Nachweis eines Toxins im Kartoffelknollengewebe nach Inokulation mit *Phytophthora infestans* (Mont.) de Bary. Potato Res. 28:193-201.

Stolle, K., and Schöber, B. 1985b. Wirkung eines Toxins von *Phytophthora infestans* (Mont.) de Bary auf Kartoffelblatter. Potato Res. 28:389-402.

Zanetti, A., Beauvais, F., Huet, J. C. and Pernollet, J. C. 1992. Movement of elicitins, necrosis-inducing proteins secreted by *Phytophthora* sp., in tobacco. Planta 187:163-170.