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ELICITIN PRODUCED BY AN ISOLATE OF *PHYTOPHTHORA PARASITICA* PATHOGENIC TO TOBACCO

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Key Word Index—Amino acid sequence; elicitin; phytopathogenic; tobacco: *Phytophthora parasitica* var. *nicotianae*.

Abstract—Elicitin was prepared from a *Phytophthora parasitica* culture isolated from tobacco in Australia. The protein with elicitor activity was purified and sequenced. This protein, which contains 98 amino acid residues, shows full homology with an elicitin produced by a completely unrelated isolate from carnation, indicating conservation of elicitin sequence within a single species.

INTRODUCTION

In culture, several *Phytophthora* species secrete low molecular mass proteins called elicitins [1]. They all have the same size (98 amino acids) and highly homologous sequences [1–5]. These fungal proteins activate defence mechanisms in tobacco (i.e. accumulation of phytoalexins and PR1a protein), cause cell necrosis and induce protection against a subsequent inoculation with the tobacco pathogen, *Phytophthora parasitica* var. *nicotianae* (*Ppn*), the causal agent of black shank disease [6–9].

The only *Phytophthora* isolates able to infect tobacco (*Ppn*) belong to *P. parasitica*. This species also encompasses isolates pathogenic to other plants than tobacco. The primary structure of the elicitin produced by one isolate of *P. parasitica* from carnation in France has been determined; this protein has been called parasiticein [3].

Tobacco isolates are unique in that they do not produce any elicitin [10]. Because elicitins are specific active elicitors on *Nicotiana* spp., it was hypothesized that lack of elicitin production could be related to pathogenicity on tobacco [10, 11]. Recently, some tobacco isolates from Australia and Zimbabwe were found to produce an elicitin [12]. This observation raised the question whether this protein would differ from the parasiticein secreted by the non-tobacco isolates of *P. parasitica*.

In this paper, we report the primary structure of the elicitin produced by the Australian tobacco isolate 310 of *P. parasitica*.

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RESULTS AND DISCUSSION

Elicitin 310 was purified from the culture filtrate of the Australian tobacco isolate 310 by ammonium sulphate precipitation followed by ion exchange chromatography by means of techniques described previously [10, 13]. This protein fraction was applied on to a DEAE-cellulose column. Elicitin 310 was not eluted with 50 mM potassium phosphate buffer, pH 6, but with 10 mM potassium phosphate buffer, pH 7, containing 1 M NaCl [Fig. 1a, peak II] in a yield of 98 mg ml^{-1}.

Elicitin 310 exhibited on SDS–PAGE a molecular weight of *ca* 10×10^3 which is similar to the molecular weight of elicitins described previously [1, 2] and reacted with a rabbit antiserum raised against cryptogein, a member of the elicitin family [2]. This protein was purified by HPLC [Fig. 1b inset] and analysed by positive FAB-MS. The spectrum showed a $[M+H]^+$ peak at m/z 10349 with an accuracy of ± 2 amu, indicating a M_r of $10\,348 \pm 2$.

Automated Edman degradation of S-carboxymethylated elicitin 310 allowed the identification of the first 25 amino acids. S-carboxymethylated elicitin 310 was cleaved with trypsin and Asp-N endoproteinase. The sequencing data of the S-carboxymethylated N-terminal end and the derived peptides, T and D, for tryptic and Asp-N cleavage products, respectively, are shown in Fig. 2. The alignment of the whole sequence of elicitin 310 is identical to that published for the parasiticein purified from a carnation isolate of *P. parasitica* [3], except for the replacement of the serine at position 65 by a threonine in elicitin 310. Recently, Kamoun *et al.* [11] cloned an elicitin gene from *P. parasitica*; the deduced amino acid

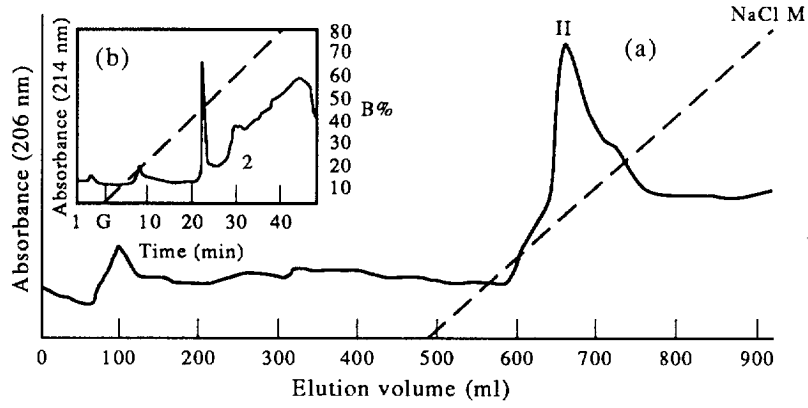

Fig. 1(a). Ion-exchange chromatography of the fraction (unbound to CM-cellulose and bound to DEAE-cellulose in 50 mM potassium phosphate, pH 6) isolated from the culture filtrate of *Phytophthora parasitica* (isolate 310) on DEAE-cellulose in 10 mM potassium phosphate, pH 7. Elicitin 310 (peak II) was eluted with a linear (0–1 M) sodium chloride gradient (broken line). (b) Inset: reverse-phase HPLC of elicitin 310 on a C4 column eluted with a gradient (broken line) of solvent B (0.085% of acetic acid in i-PrOH–water, 4:1) in solvent A (0.1% of trifluoroacetic acid in water).

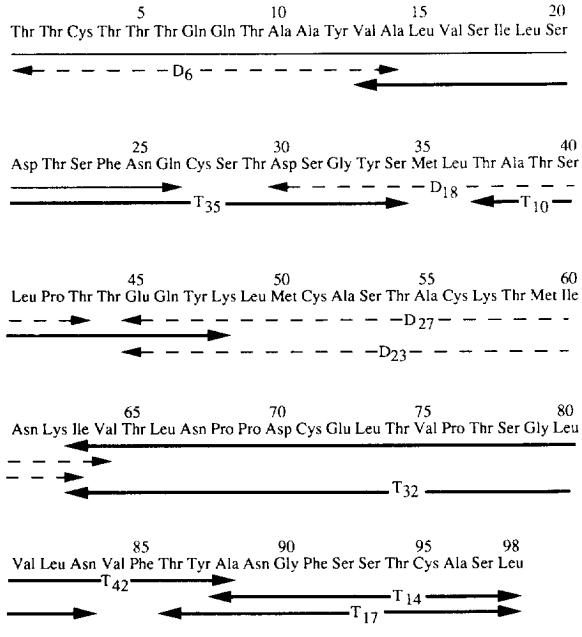

Fig. 2. Complete amino acid sequence of elicitin 310. (→) N-terminus of the S-carboxymethylated protein; (↔) cleaved peptides (T, tryptic peptides; D, endoproteinase Asp-N-cleaved peptides).

sequence matches completely that of elicitin 310. In order to determine whether a point difference might occur between elicitins produced by different isolates in a single *Phytophthora* species, the elicitin produced by the same carnation isolate 26 used by Nespoulos *et al.* [3] was purified and sequenced (not shown). This sequence was found to be identical to that of elicitin 310, including a threonyl residue at position 65.

The sequencing of elicitin 310 gave 98 amino acids resulting in a molecular mass of 10 355.75 calculated for an elemental formula $C_{446}H_{716}O_{153}B_{110}S_9$. This value was nearly similar to that of the M_r calculated for other elicitins (Table 1). Like capsicein, this molecule is an acidic protein with a pI of 4.7, whereas cryptogein and cinnamomin have higher pIs [1, 2].

In accordance with the sequence data, the locations of the six cysteine residues in the polypeptide chain have been determined to be at positions 3, 27, 51, 56, 71 and 95. Three interchain disulphide bridges are to be expected for the native protein resulting in a M_r of 10 349.70. The M_r is in good agreement with the result of the mass spectrum.

Applied to tobacco plants, elicitin 310 induced leaf necrosis (at 400 μg plant^{-1}) and reduced the ability of a black shank isolate (183) to colonize the stem (already at 100 μg plant^{-1}). These results were identical to those obtained with parasiticein from isolate 26 compared in the same test.

Our results demonstrate that the elicitin excreted by isolate 310 from tobacco in Australia has exactly the same sequence as the parasiticein produced by isolate 26 from carnation in France. As these isolates belong to different haplotypes, determined by mitochondrial DNA RFLP analysis [14], this is a strong indication of a total conservation of the elicitin produced within the species *P. parasitica*. The exact sequence of this protein corresponds to that deduced from the *Par A*1 gène cloned by Kamoun *et al.* [11] rather than that previously published [3].

The finding that an isolate able to cause tobacco black shank disease produces the same parasiticein (with the same elicitor activity) as a non-tobacco isolate implies that other factors are involved in determining host-specificity to tobacco in *P. parasitica*. Among black shank isolates, however, in comparative inoculations, parasiticein-producing ones never reached the highest virulence of those lacking elicitin production [12], suggesting that parasiticein interferes with tobacco colonization.

Table 1. M_r determinations

Elicitin	Elementary formula	Calculated M_r	
		*	†
Elicitin 310	$C_{446}H_{716}O_{153}N_{110}S_9$	10355.75	10349.70
Cryptogein	$C_{446}H_{721}O_{147}N_{115}S_9$	10334.85	10328.80
Capsicein	$C_{439}H_{703}O_{190}N_{103}S_9$	10166.60	10160.55
Cinnamomin	$C_{445}H_{718}O_{148}N_{112}S_9$	10293.80	10287.75

*In accordance with the sequencing data.
†For native protein with three disulphide bridges.

EXPERIMENTAL

Cultures. Phytophthora parasitica isolate 310, from tobacco plants grown in Queensland (Australia), was a gift from Dr Guest. Isolate 26 was isolated from carnation at INRA Antibes, France. Both isolates were grown in Plich liquid medium [15] as previously described [16]. The elicitin non-producing tobacco isolate 183 was maintained on malt agar medium and used for plant inoculations.

Extraction and purification of elicitin. The technique was as previously described by Ricci *et al.* [10].

PAGE and immunoblotting. SDS–PAGE of denatured protein was performed on 15% polyacrylamide gels at pH 8.3 according to Laemmli [16]. Gels were stained with 0.1% Coomassie Brilliant Blue. Alternatively, the gels were used for immunoblotting according to the method of Towbin *et al.* [17].

FAB-MS. Prior to FAB-MS analysis elicitin was desalted by means of reverse-phase HPLC on a C4 Vydac column (0.46 × 25 cm). Elicitin was eluted with a linear gradient (0–80%) of solvent B (0.085% trifluoroacetic acid in i-PrOH–H_2O, 4:1) in solvent A (0.1% trifluoroacetic acid in H_2O) over 75 min.

All FAB mass spectra were recorded in the positive ion mode on a VG ZAB2 SEQ mass spectrometer. The instrument was equipped with a standard VG FAB source with a caesium ion gun delivering *ca* 2 μA of caesium ion current with *ca* 40 kV energy.

Spectra were obtained with a magnet scan rate of 20 sec per decade using a magnet of mass range 13000 (at 8 kV). The mass range was calibrated using caesium iodide clusters. The spectra were collected and processed using the VG 11-250 J data system.

Samples were dissolved in H_2O–HOAc (19:1). The sample (2 μl) was deposited on the FAB probe tip mixed with matrix, 3-nitro-benzyl alcohol.

Reduction and S-carboxymethylation of elicitin. Elicitin 310 was reduced with dithiothreitol and S-carboxymethylated with unlabelled iodoacetic acid according to Fernandez-Luna *et al.* [18].

Elicitin cleavage and peptide separation. Asp-N-endoproteinase (Boehringer, Germany) was used for the cleavage of 0.2 mg elicitin with an enzyme:substrate ratio of 1:100 (wt:wt) for 18 hr at 37° in 50 mM sodium phosphate, pH 8.0.

Tryptic peptides were obtained using S-carboxymethylated protein dissolved in 0.2 M N-ethylmorpholine acetate, pH 8.2 (10 mg ml^{-1}) and incubated with trypsin (Sigma) treated with L-1 (tosylamido)-2-phenylethylchloromethylketone [enzyme:substrate ratio 1:100 (w:w)] for 3 hr at 37°. The digestion was stopped by lowering the pH to 2–3 with 6 M HCl.

Fractionation of peptides by reversed phase HPLC was performed with a C8 Aquapore RP 300 Brownlee column (0.46 × 22 cm). Peptides were eluted with a linear gradient from 5 to 65% solvent B (0.09% trifluoroacetic acid in MeCN–H_2O, 4:1) in solvent A (0.1% trifluoroacetic acid in H_2O) for 60 min at a flow rate of 1 ml min^{-1}. The peptides were detected by UV absorption at 214 nm and collected manually.

Amino acid sequencing. Automated Edman degradation was performed using a 470 A Applied Biosystems gas-phase sequencer. Whole protein or peptide samples were spotted on a polybrene precycled glass-fibre disc. Phenylthiohydantoins (PTH) amino acids, were identified on line with a 120 A Applied Biosystems PTH-Analyser.

All the products and reagents used for sequencing were from Applied Biosystems (U.S.A.). The sequencing was performed using the O3R PTH program with slight modifications. Chemical delivery rates were carefully monitored during the sequence determination.

Elicitor activity of elicitin on tobacco. Elicitor assays were performed as described previously [10].

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