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Structure-function relationships of α and β elicitins, signal proteins involved in the plant-*Phytophthora* interaction

Claude Nespoulous, 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

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Abstract. Elicitins form a family of 10-kDa holoproteins secreted by various *Phytophthora* species. The large-scale purification of parasiticein, a novel elicitin secreted by *P. parasitica*, led to the determination of its sequence. We have compared the necrotic activities and the primary and secondary structures (determined through circular dichroism) of four elicitins. On tobacco plants, they could be classified into two classes: α, comprising capsicein and parasiticein (less necrotic), and β, comprising cryptogein and cinnamomin (very toxic with a necrosis threshold of 0.1 μg per leaf). The features of elicitin structure which might be involved in the interaction of elicitins with the leaf target cells and that could explain the different necrosis-inducing properties of the two proteins are investigated. About 75% sequence identity was observed between the four elicitins: only two short terminal regions are heterologous, while the central core is mainly conserved. The circular-dichroism spectra showed that the secondary structure of the elicitins was largely conserved. All of them consisted of approx. 50% α-helix with little or no β-structure. Comparisons of the complete sequences, amino-acid compositions, isoelectric points, hydropathy indices and the secondary-structure predictions correlated with the necrotic classification. Alpha elicitins corresponded to acidic molecules with a valine residue at position 13, while β elicitins were basic with a lysine at this position, which appeared to be a putative active site responsible for necrosis induction.

Key words: Amino acid (sequence) – Elicitor – Phytopathogen – *Phytophthora* – Protein (secondary structure)

Introduction

In culture, *Phytophthora cryptogea, P. cinnamomi, P. capsici* and *P. parasitica* secrete proteins of low relative molecular mass (M_r) (Bonnet et al. 1985), namely cryptogein, cinnamomin, capsicein and parasiticein, respectively, which have been purified (Billard et al. 1988), even on a large scale (Huet et al. 1990). These fungal proteins belong to a novel protein family, called elicitins, and are different from other known proteins, not only those of fungal origin such as the *Phytophthora megasperma* Pmg elicitor (Parker et al. 1988), a *Phytophthora* Ppn 46e protein (Farmer and Helgeson 1987), a glycoprotein isolated from cell walls of *Puccinia graminis* (Beissmann and Reisener 1990), and a *Cladosporium* necrosis-inducing peptide (Schottens-Toma and De Wit 1988), but also any defense-related protein in higher plants (Bowles 1990). Elicitins act as fungal signals in the plant-*Phytophthora* interactions and are responsible for the tobacco incompatible reaction. When applied to tobacco plants, elicitins induce systemic remote leaf necrosis and also give rise to protection against a subsequent inoculation with the tobacco pathogen *P. nicotianæ* (Ricci et al. 1989). Elicitins from various fungal species exhibit different levels of biological activity: cryptogein and cinnamomin cause visible leaf necrosis when applied at approx. 1 μg per plant, whereas 50-fold as much capsicein or parasiticein is required for the same reaction; in contrast to cryptogein and cinnamomin, capsicein and parasiticein induce protection even in the near absence of leaf necrosis (Ricci et al. 1989). The complete amino-acid sequences of cryptogein, capsicein and cinnamomin are known (Huet and Pernollet 1989). They are homologous proteins (homology > 80%) of 98 residues, devoid of glycosylation. The differences found among the three known elicitin sequences have been correlated with their biological activities. Among these differences, only two lysines, located at the amino- and carboxy-terminal regions, were predicted to be the key amino acids involved in the differential control of protection with respect to necrosis (Huet and Pernollet 1989).

* To whom correspondence should be addressed

Abbreviations: CD = circular dichroism; RPLC = reversed-phase liquid chromatography; SDS–PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

We have therefore determined the amino-acid sequence of parasiticein to obtain a more precise knowledge of the amino-acid residues that are involved in the modulation of necrotic potential. Furthermore, using far-UV circular dichroism, we undertook a preliminary study of the secondary structures of the four different molecules, in order to determine whether the amino-acid replacements indeed alter significantly their conformation. The structural features were found to be correlated with the ability of different elicitins to induce necrosis on detached tobacco leaves. In the migration of cryptogein and capsicein in the plant, however, no differences could be found (data not shown).

Materials and methods

Elicitin purification. Phytophthora cryptogea (isolate A52), *P. cinnamomi* (isolate V280), *P. capsici* (isolate A147) and *P. parasitica* (isolate A26) from the *Phytophthora* collection at INRA Antibes and Versailles, France, designated A and V, respectively, were grown on Plich liquid medium (Plich and Rudnicki 1979) without yeast extract and peptone. Two liters of culture filtrates were sterilized by filtration through a 0.22-μm (GV) Millipore-membrane (Millipore-France, St Quentin en Yvelines) before chromatography. Elicitins were purified using a two-step chromatographic procedure: ion-exchange chromatography and gel filtration for cryptogein and cinnamomin. Capsicein and parasiticein, which are both acidic, were submitted to reversed-phase liquid chromatography (RPLC) in ammonium-acetate buffer before gel filtration.

Ion-exchange chromatography was adapted from Hou and Mandaro (1986) as follows. Two liters of culture filtrate were loaded on four SulfoPropyl Zeta-Prep-disks (model 60; Flot-Cuno, Boissy-St Léger, France) using a Minipuls II Gilson peristaltic pump (Gilson-France, Villiers-le-Bel) at a flow rate of 2.5 ml · min^{-1} at room temperature (22° C). The loading of the filtrate and the elution of cryptogein and cinnamomin were monitored with an ISCO UA 5 photometer (Roucaire, Vélizy, France) at 280 nm. Disks were equilibrated in 10 mM Na-acetate (pH 4) and eluted with the same buffer, but containing 0.5 M NaCl.

Capsicein and parasiticein were subjected to RPLC using a 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. 1 l), adjusted to pH 7.0, were loaded using the high-pressure pump onto two Aquapore (C8) RP 300 cartridges (30 mm long, 10 mm i.d.) at 2.5 ml · min^{-1} per cartridge (Brownlee, Applied Biosystems-France, Roissy). Elution was carried out at room temperature (22° C- using a gradient of CH_3CN (FISONS, far-UV grade; Touzart et Matignon, Vitry-sur-Seine, France) obtained with the 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 14% CH_3CN in 20 min, from 14 to 27.5% in 22.5 min, 27.5% hold for 10 min, from 27.5–32% in 7.5 min, washing for 5 min at 50% CH_3CN, followed by a 20-min re-equilibration at 5% – was applied at a flow rate of 2.5 ml · min^{-1}. Elution was monitored through absorbance at 275 nm.

The second common purification step consisted of exclusion-diffusion chromatography, performed on a column (450 mm long; 32 mm i.d.) made of G50 fine Sephadex (Pharmacia-France, St Quentin en Yvelines) in MilliQ water (Millipore-France, St Quentin en Yvelines) at 4 ml · min^{-1} with the same equipment as that used for ion-exchange chromatography. All elicitins were lyophilized before use. Elicitin purity was checked using a peptide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) system using standards from the Pharmacia PMW calibration kit (Sallantin et al. 1990).

Tests for necrotic activities. Purified elicitins were tested on approx. 60-d-old plants of tobacco (*Nicotiana tabacum* L., cv. Xanthi) cultivated in a greenhouse. Six doses of each elicitin (1, 10, 100 ng, 1, 10 and 100 μg in 10 μl of pure water) were applied to still expanding detached leaves (approx. 20-cm long). The drop of elicitin solution was applied to the petiole cut end (length of the petiole approx. 15 mm) and was allowed to be totally absorbed into the petiole which was then dipped into a nutritive solution (adapted from Baudet et al. 1986). Elicitin-treated leaves were kept at room temperature in the dark for several days to allow the necrotic symptoms to develop. The results presented (number of leaves exhibiting necrotic symptoms) were obtained after 2 d when the symptoms were stable. Experiments were done in quadruplicate.

Reduction and alkylation of parasiticein. For N-terminal analysis, the reduction and alkylation was conducted directly on the sequencing membrane according to Yuen et al. (1989). For other purposes, parasiticein was reduced with 2-mercaptoethanol and alkylated either with iodoacetic acid or 4-vinyl-pyridine according to Henschen (1986) before submission to enzymatic or chemical cleavage. The water-insoluble material obtained was desalted by five successive water washings (2 ml of H_2O per mg of protein).

Digestion with CNBr and isolation of digested peptides. Reduced and alkylated parasiticein was dissolved (1 mg · ml^{-1}) in 70% (v/v) formic acid with cyanogen bromide (10 mg · ml^{-1}). The mixture was incubated for 24 h at room temperature in the dark; the reaction mixture was then diluted five-fold with cold distiled water and lyophilized. The CNBr peptides were separated either by exclusion-diffusion chromatography on a column (500 mm long, 9 mm i.d.) of Fractogel (Merck-France, Nogent-sur-Marne) HW40S at a flow-rate of 0.2 ml · min^{-1} of 30% CH_3CN in 0.1% CF_3COOH in water, or by RPLC. The RPLC fractionation of peptides was conducted with an RP 300 Octyl Aquapore (30 mm long, 4.6 mm i.d.) column at room temperature on an SP 8800 Spectra Physics apparatus with a Jasco M320 UV diode array detector (Prolabo, Paris, France) and a Pharmacia Frac 100 collector. Solvent A was Millipore-treated water with 0.1% CF_3COOH, and solvent B was 40% Millipore-treated water, 60% CH_3CN and 0.1% CF_3COOH. The following gradient was used at a flow-rate of 0.5 ml · min^{-1}: from 100% of solvent A to 100% of solvent B in 60 min, 5 min at this constant concentration, from 100% B to 100% A in 10 min, and 10 min of re-equilibration at 100% of solvent A.

Tryptic digests of parasiticein and isolation of digested peptides. Reduced and alkyled parasiticein, desalted by RPLC, was dissolved (1 mg · ml^{-1}) in 0.2 M ammonium-hydrogen-carbonate (pH 8.0) buffer before digestion with TPCK trypsin (trypsin, EC 3.4.21.4); Sigma ref T8642, batch number 38F8140) for 18 h at 22° C (enzyme-to-substrate weight ratio 1/50). The digestion was stopped by acidification to pH 2–3 with 10% CF_3COOH. Separation by RPLC was conducted as described above for CNBr peptides.

Tryptic digests of CNBr-cleaved parasiticein and isolation of digested peptides. Peptides obtained after digestion with both CNBr and trypsin were separated as described above.

Protein sequencing. Automated Edman degradation of the whole protein and of peptides was performed using an Applied Biosystems 475A sequencer and its online phenylthiohydantoin amino-acid (Pth-Xaa) analyzer (model 120A); the reagents and methods were as specified by the manufacturer (Applied Biosystems-France, Roissy).

Circular dichroism. Circular dichroism (CD) spectra were recorded at 1-nm intervals over the wavelength range 180–300 nm, using a Mark V dichrograph (Jobin-Yvon, Longjumeau, France). The

dichrograph was calibrated using an iso-androsterone (Roussel-Uclaf, Romainville, France) solution (1.3 mg · ml^{-1} in dioxane). The optical rotation was checked with horse-heart cytochrome *c* (Fluka), chicken-egg-white lysozyme (Sigma) and rabbit-muscle L-lactate dehydrogenase (Fluka). The CD measurement were made at room temperature. Protein samples (0.1 mg · ml^{-1} in water) were placed in a 0.2-cm-pathlength cell. Elicitin concentrations were determined spectrophotometrically using an extinction coefficient of 7680 M^{-1} · cm^{-1} at 277 nm (wavelength of the elicitin relative maximum in this near UV-region) for cryptogein, capsicein and cinnamomin, and 6230 M^{-1} · cm^{-1} at 277 nm for parasiticein, deprived of one tyrosine residue. Five reproducible spectra were collected for each sample. The net spectrum of each protein was obtained by subtracting the base line obtained with pure water. The CD data were expressed as the mean residue ellipticity and the spectra analyzed following the method of Yang et al. (1986) for the determination of the contribution of the different secondary structures. This method is based on the least-squares fitting of the measured spectrum, adjusted to the weighted sum of pure spectra corresponding to the different secondary structures, spectra deduced from known three-dimensional structures of proteins. The method was tested with the same proteins as mentioned above. The results are given with their confidence limits ($P=0.95$) obtained after curve-fitting computation.

Homology search in data libraries. Search for homologies between elicitins and other proteins was performed using the method of Kanehisa (1984) with the following libraries: National Biomedical Research Foundation (release 26) and the translation of the open reading frames of GENBANK (release 65) and the European Molecular Biology Library (release 24+) available at the French data base BISANCE (Dessen et al. 1990). All computations were done using the similarity matrix of Dayhoff et al. (1978).

Structural predictions. Secondary-structure predictions were established using the GOR algorithm of Garnier et al. (1978). This algorithm may be parameterized with decision constants depending on the structural type of the studied protein or chosen for the best fit of the secondary-structure proportions obtained through CD measurements. Hydropathy profiles were done using the method of Kyte and Doolittle (1982).

Results

Elicitin purification. With the purification procedure used here adapted from Huet et al. (1990), large amounts of parasiticein could be rapidly collected in a pure form, due to the additional RPLC step that was omitted in the earliest preparations (Billard et al. 1988). As for the other elicitins (Huet et al. 1990), no contaminant protein was revealed through SDS–PAGE or isoelectric focusing after silver staining (not shown). The parasiticein M_r, measured by SDS–PAGE, was found to be 10000. Atmospheric-pressure ionization mass spectrometry was used to check the purity and the molecular size of the elicitins. No contamination by any atom or molecule larger than Na^+ was detected. Values of 10327 ± 2, 10289 ± 4 and 10165 ± 1 were obtained for cryptogein, cinnamomin and capsicein, whereas expected molar masses from the sequence were 10329, 10288 and 10161, respectively. These values confirmed that all cysteine residues were disulfide-bonded, and the absence of glycosylation (Ricci et al. 1989).

Comparison of necrosis elicitation by the four elicitins. Purified cryptogein and cinnamomin elicited the necrosis of detached leaves in amounts as low as 0.1 μg per leaf (Fig. 1). Necrosis induced on the various leaves by the elicitins was detectable from 24 h onwards and did not evolve after 2 d. A comparable necrosis induction was obtained with amounts of capsicein and parasiticein approx. 50–100 times higher than those of cryptogein and cinnamomin.

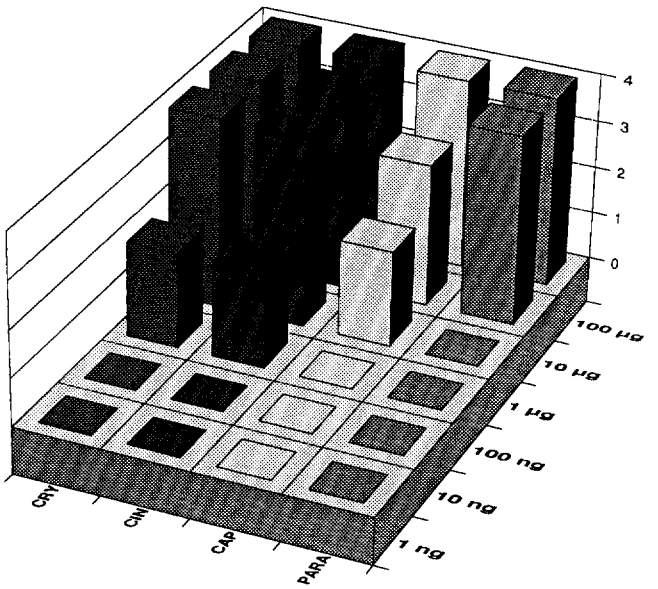

Fig. 1. Comparison of necrosis induction by various elicitins on tobacco detached leaves. Elicitins were tested on 60-day-old tobacco plants (cv. Xanthi) cultivated in a greenhouse. Six different doses of each elicitin diluted in 10 μl of pure water were applied to detached leaves kept at room temperature in the dark for 48 h. *CRY*, cryptogein; *CIN*, cinnamomin; *CAP*, capsicein; *PARA*, parasiticein. *Ordinates* indicate the number of tobacco leaves exhibiting obvious necrotic areas. Experiments were done in quadruplicate

Isolation and sequencing of parasiticein peptide fragments. Several enzymatic and chemical digestions were needed to obtain overlapping peptides. Figure 2 shows the separation of the peptides that were sequenced. They were obtained either after digestion with trypsin, CNBr cleavage, or combined trypsin and CNBr cleavage. The peptides were separated by RPLC; they are named according to Fig. 2 (only those useful for the determination of the whole sequence are named with a letter). With an initial sequencing yield of 53%, the pyridylethylated N-terminal end of parasiticein was sequenced up to Thr 39. The alignment of the sequenced peptides of parasiticein is shown in Fig. 3. The CNBr peptide B2 (Fig. 2C) allowed the alignment of the N-terminus with the double-digested peptide BT2 (Fig. 2D). It was aligned with the tryptic peptides T (Fig. 2A) using the CNBr peptide B3 (Fig. 2B). The peptide B1 (Fig. 2C) was joined to the peptide B3 due to the peptide T and allowed the overlap with the C-terminal end found in the peptide BT1 (Fig. 2D). No microheterogeneity was observed in the phenylthiohydantoin amino-acid in the course of parasiticein sequencing.

Comparison of elicitin features. Comparisons of the amino-acid compositions and molecular parameters of the

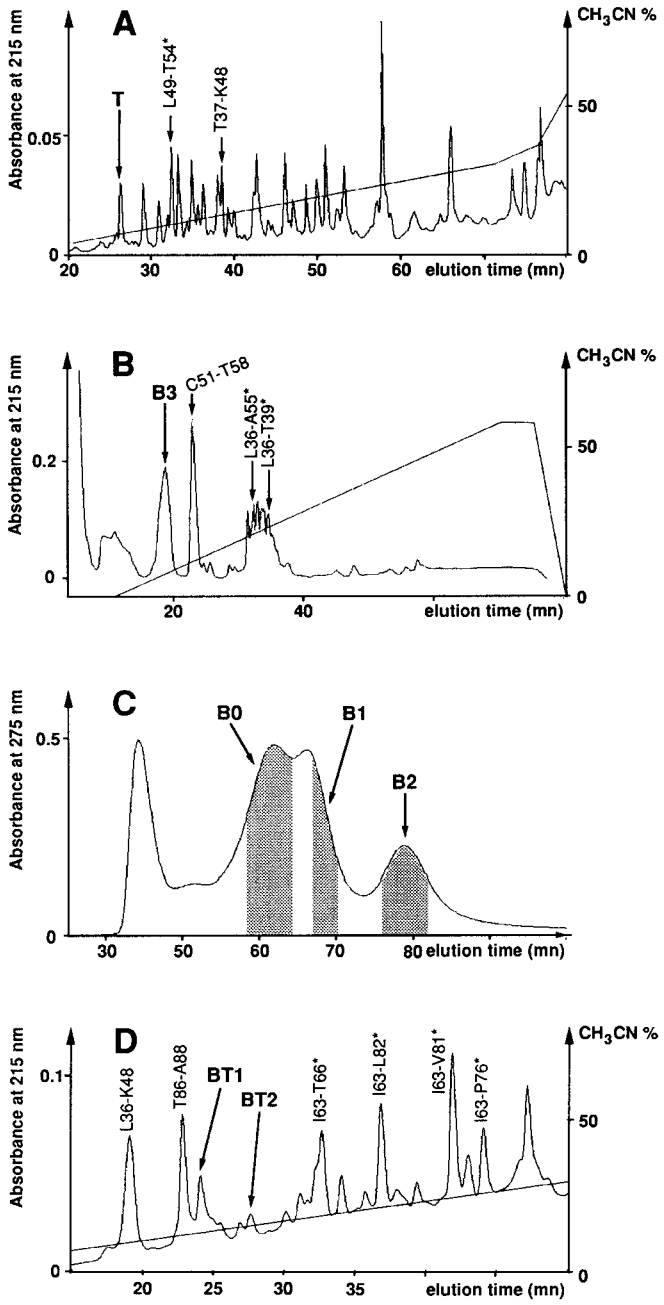

Fig. 2A–D. Reversed-phase liquid chromatography of peptides resulting from parasiticein cleavage (peptide maps). Peptides are identified on chromatograms using their first- and last- identified amino-acid residues, numbered according their position in the sequence, except those useful for the determination of the whole sequence which are named by letters (*T*, peptide obtained after proteolysis with trypsin; *B*, CNBr-cleaved peptides, *BT*, peptides obtained after digestion of the CNBr peptide B0 with trypsin). *Stars* indicate that the sequencing process was stopped before achieving the end of the peptide molecule. The *straight lines* represent the CH_3CN gradient. **A** Separation by RPLC of peptides obtained after TPCK trypsin cleavage; the peptide T was used for sequence alignment. **B** Separation by RPLC of CNBr-cleaved peptides; the peptide B3 was used for sequence alignment. **C** Exclusion-diffusion chromatography on Fractogel HW40S of CNBr-cleaved peptides; peptides B1 and B2 were directly sequenced, B0 submitted to digestion with trypsin. **D** Separation by RPLC of tryptic peptides resulting from the trypsin cleavage of B0; BT1 and BT2 were used for sequence alignment

four elicitins revealed the similarity between elicitins: they had the same number of residues (98) and a similar molecular weight, calculated using the mean isotopic composition. Assuming the occurrence of three disulfide bridges as in other elicitins, parasiticein is a 10350-Da protein, instead of 10329 for crytogein, 10288 for cinnamomin and 10161 for capsicein. The four elicitins share in common ten Leu, three Ile, four Pro, four Gln, six Cys, three Met and three Gly residues. Like cryptogein, cinnamomin and capsicein, parasiticein lacks three amino acids (Trp, His, Arg). For all these molecules, Leu, Ser, Thr and Ala accounted for nearly half of the residues. Among the four elicitins so far sequenced, parasiticein is the only one which exhibits four Tyr residues instead of five, compensated by an extra Phe, resulting in a lower extinction coefficient (6230 instead of 7680 $M^{-1} \cdot cm^{-1}$ at 277 nm, the wavelength of the elicitin maximum). Owing to a low proportion of lysine residues, parasiticein was much more comparable to capsicein than to the two other elicitins. Its calculated isoelectric point was 4.70, close to that of capsicein (4.54), whereas cryptogein (8.49) and cinnamomin (7.85) were basic.

Comparison of elicitin sequences. No deletion was necessary for aligning the sequences of the four elicitins (Fig. 4A). The percentage match between them was 74.5%. Parasiticein and cinnamomin exhibited an 88.8% exact pairing, cinnamomin and cryptogein 87.8%. But less than 83% strict homology was found between parasiticein with either cryptogein or cinnamomin, and between cinnamomin with either parasiticein or capsicein. The elicitin consensus sequence (Fig. 4B) showed only 25 positions that underwent replacements. The most conserved part of the sequence was found to be the central core in which the replacements occurred only once or were highly conservative in terms of three-dimensionnal structure (Dayhoff et al. 1978). Figure 4A shows that only eight positions (2, 5, 13, 14, 61, 65, 72 and 94) were correlated with differences in necrotic activities.

Circular-dichroism spectra of elicitins. Figure 5 shows the far-UV CD spectra of the four elicitins. Although not strictly identical, they are all very similar and typical of the α-helix. The CD spectra were analyzed following the method of Yang et al. (1986) for the determination of the contribution of the different secondary structures, which are reported in Table 1. Elicitins appeared to be rich in α-helices (about half of the molecule) and poor in or devoid of β-sheets. Only cinnamomin exhibited a lower proportion of α-helix, as indicated by the peculiar shape of its CD spectrum.

Secondary-structure prediction and buried areas of elicitins. Since the GOR algorithm can be parameterized (Garnier et al. 1978), secondary-structure predictions were made using decision constants optimized to fit the values obtained through CD measurements (i.e. −80 for α-helix and 310 for β-structure). As shown in Table 1, a good agreement between measurements and prediction

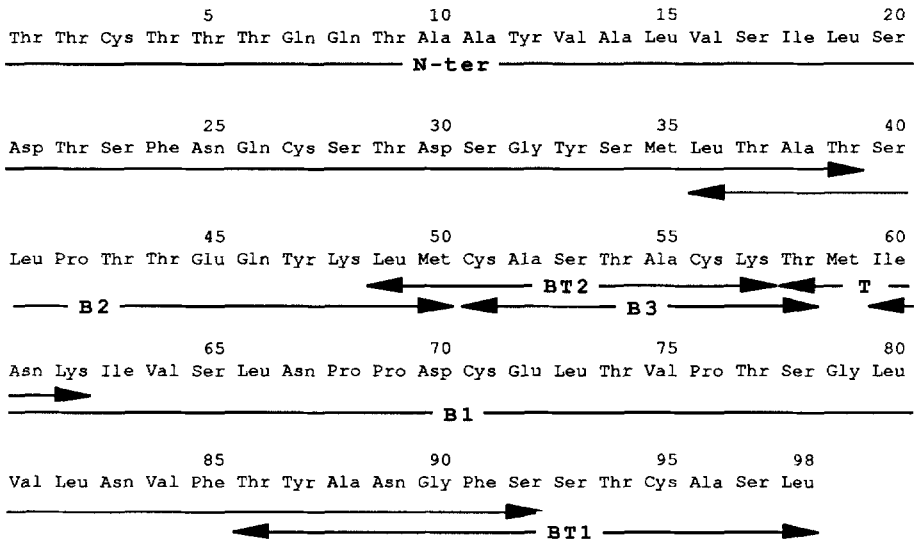

Fig. 3. Amino-acid sequence of parasiticein. Peptides are named according to Fig. 2. →: N terminus of the in-situ-pyridylethylated protein. ↔: Cleaved peptides (*T*; tryptic peptides: *B*, CNBr-cleaved peptides; *BT*, peptides resulting from a double cleavage with CNBr and trypsin)

A. Sequence comparison

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                    5                  10                 15                  20
Cry  → Thr Ala Cys Thr Ala Thr Gln Gln Thr Ala Ala Tyr Lys Thr Leu Val Ser Ile Leu Ser
Cin  → Thr Ala Cys Thr Ala Thr Gln Gln Thr Ala Ala Tyr Lys Thr Leu Val Ser Ile Leu Ser
Cap  → [Ala] [Thr] Cys Thr [Thr] Thr Gln Gln Thr Ala Ala Tyr [Val Ala] Leu Val Ser Ile Leu Ser
Para → Thr [Thr] Cys Thr [Thr] Thr Gln Gln Thr Ala Ala Tyr [Val Ala] Leu Val Ser Ile Leu Ser
                   25                 30                 35                  40
Cry  → Asp [Ala] Ser Phe Asn Gln Cys Ser Thr Asp Ser Gly Tyr Ser Met Leu Thr Ala [Lys] Ala
Cin  → [Glu] Ser Ser Phe [Ser] Gln Cys Ser [Lys] Asp Ser Gly Tyr Ser Met Leu Thr Ala Thr Ala
Cap  → Asp Ser Ser Phe Asn Gln Cys [Ala] Thr Asp Ser Gly Tyr Ser Met Leu Thr Ala Thr Ala
Para → Asp [Thr] Ser Phe Asn Gln Cys Ser Thr Asp Ser Gly Tyr Ser Met Leu Thr Ala Thr [Ser]
                   45                 50                 55                  60
Cry  → Leu Pro Thr Thr Ala Gln Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys Asn Thr Met Ile
Cin  → Leu Pro Thr [Asn] Ala Gln Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys Asn Thr Met Ile
Cap  → Leu Pro Thr Thr Ala Gln Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys Asn Thr Met Ile
Para → Leu Pro Thr Thr [Glu] Gln Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys [Lys] Thr Met Ile
                   65                 70                 75                  80
Cry  → Lys Lys Ile Val [Thr] Leu Asn Pro Pro Asn Cys Asp Leu Thr Val Pro Thr Ser Gly Leu
Cin  → Lys Lys Ile Val [Ala] Leu Asn Pro Pro [Asp] Cys Asp Leu Thr Val Pro Thr Ser Gly Leu
Cap  → [Thr] Lys Ile Val Ser Leu Asn Pro Pro Asp Cys [Glu] Leu Thr Val Pro Thr Ser Gly Leu
Para → [Asn] Lys Ile Val Ser Leu Asn Pro Pro Asp Cys [Glu] Leu Thr Val Pro Thr Ser Gly Leu
                   85                 90                 95          98
Cry  → Val Leu Asn Val Tyr Ser Tyr Ala Asn Gly Phe Ser [Asn] Lys Cys [Ser] Ser Leu
Cin  → Val Leu [Asp] Val Tyr [Thr] Tyr Ala Asn Gly Phe Ser Ser Lys Cys Ala Ser Leu
Cap  → Val Leu Asn Val Tyr Ser Tyr Ala Asn Gly Phe Ser [Ala] [Thr] Cys Ala Ser Leu
Para → Val Leu Asn Val [Phe] [Thr] Tyr Ala Asn Gly Phe Ser Ser [Thr] Cys Ala Ser Leu
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B. Consensus sequence

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                    5                  10                 15                  20
Thr Ala Cys Thr Ala Thr Gln Gln Thr Ala Ala Tyr Lys Thr Leu Val Ser Ile Leu Ser
Ala Thr         Thr                                Val Ala
                   25                 30                 35                  40
Asp Ser Ser Phe Asn Gln Cys Ser Thr Asp Ser Gly Tyr Ser Met Leu Thr Ala Thr Ala
Glu Thr         Ser         Ala Lys                                    Lys Ser
    Ala
                   45                 50                 55                  60
Leu Pro Thr Thr Ala Gln Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys Asn Thr Met Ile
            Asn Glu                                                Lys
                   65                 70                 75                  80
Lys Lys Ile Val Ser Leu Asn Pro Pro Asp Cys Asp Leu Thr Val Pro Thr Ser Gly Leu
Thr             Ala                 Asn     Glu
Asn             Thr
                   85                 90                 95          98
Val Leu Asn Val Tyr Thr Tyr Ala Asn Gly Phe Ser Ser Lys Cys Ala Ser Leu
        Asp     Phe Ser                            Asn Thr     Ser
                                                   Ala
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Fig. 4A, B. Comparison of the primary structures of cryptogein, cinnamomin, capsicein and parasiticein. **A** Alignment of the elicitin sequences. *Cry*, cryptogein; *Cin*, cinnamomin; *Cap*, capsicein; *Para*, parasiticein. *Boxes* emphasize differences, *shaded boxes* show replacements which are correlated with the reduced necrotic activity, and the *vertical arrow* emphasizes the position most likely involved in necrosis. **B** Elicitin consensus sequence

of the periodic structure was then found. The N-terminal moiety of the elicitin molecule is predicted in two α-helix areas, separated by a region of β-turns, while the C-terminal end (beyond position 62) is more or less aperiodic or in β-turns. Only the secondary-structure predictions of the terminal ends were seriously affected by the residue replacements. The hydropathy profiles (not shown) were very similar to each other, except at the C-terminal end and around position 13. Capsicein and parasiticein profiles were very close together but clearly different from cinnamomin and cryptogein. The changes of the hydropathy index were clearly correlated with the

Table 1. Circular dichroism and predicted secondary structures of elicitins. Data obtained with CD measurements are given with their confidence limits ($P = 0.95$). Predicted values (Pred) were computed using the GOR algorithm with decision constants chosen to optimize the fitting of the global secondary-structure proportions deduced from predictions with those provided through the CD measurements (α-helix decision constant = −80 and β-structure decision constant = 310)

Elicitin	α-helix (%)		β-sheet (%)		β-turn (%)		aperiodic (%)	
	CD	Pred	CD	Pred	CD	Pred	CD	Pred
Cryptogein	49.2 ± 3.4	50	0 ± 20.1	0	22.7 ± 6.4	38	28.1 ± 3.9	12
Cinnamomin	40.1 ± 1.8	60	4.8 ± 10.8	0	18.7 ± 3.4	29	36.4 ± 2.1	11
Capsicein	53.7 ± 3.9	52	0 ± 23.4	0	21.7 ± 7.4	35	24.6 ± 4.6	13
Parasiticein	49.9 ± 3.3	47	0 ± 19.9	0	20.1 ± 6.3	34	30.0 ± 3.9	19

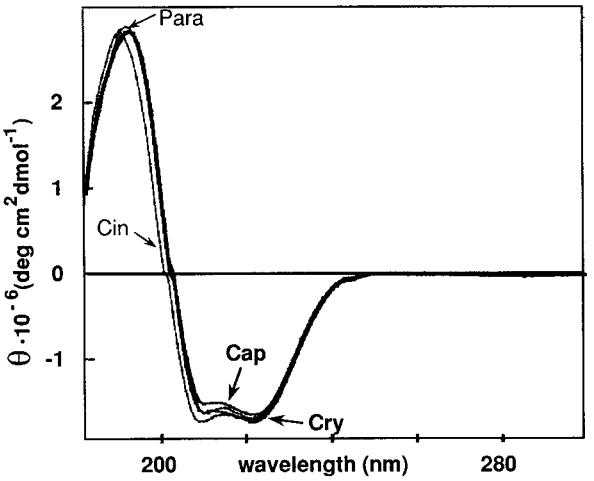

Fig. 5. Comparison of the circular-dichroism spectra of cryptogein, cinnamomin, capsicein and parasiticein. *Cry*, cryptogein; *Cin*, cinnamomin; *Cap*, capsicein; *Para*, parasiticein

elicitin class, near the N-terminus, where differences were prominent.

Discussion

The correspondence between the M_r calculated from the sequence and that measured by SDS–PAGE and confirmed by mass spectrometry shows that the side-chains of elicitins do not undergo any post-translational modifications. The M_r values found by mass spectrometry are consistent with the occurrence of three disulfide bridges per elicitin molecule, in agreement with the results obtained using Ellman's reagent showing no free sulfhydryl group (not shown). The absence of sugar in parasiticein is in agreement with the absence of N-glycosylation sites along the sequence. When parasiticein was sequenced, no unusual repetitive yield decrease, at serine or threonine positions indicating O-glycosylation, was found. On the other hand, the purity of the elicitins, checked either by silver staining after electrophoresis or isoelectric focusing, by mass spectrometry or by amino-acid sequencing, eliminates the possibility that some variable contaminating protein could be responsible for the differences in biological activities observed between elicitins. The search for homologies in protein data bases failed to reveal any significant sequence identity of parasiticein with any known protein, except elicitins.

On tobacco plants, the four elicitins tested can be classified into two classes α and β, the first one, less necrotic, comprises capsicein and parasiticein, and the second one, very toxic (necrosis threshold of 0.1 μg per leaf), comprises cryptogein and cinnamomin. The sequence homologies are consistent with the classification based on amino-acid composition and isoelectric points. Since the central core of the four molecules is highly conserved, it is probably essential for biological activity. The terminal ends, which vary from one elicitin to another, would modulate the necrotic activity differently. Neither the replacements common to elicitins of different toxicities nor those peculiar to only one elicitin would be essential for explaining the differences in activities. The only potential candidates for the control of necrotic activity were found at eight positions (shaded boxes in Fig. 4A): 2 and 5 (Ala by Thr), 13 (Lys by Val), 14 (Thr by Ala), 61 (Lys by Thr or Asn), 65 (Ser by Thr or Ala), 72 (Asp by Glu) and 94 (Lys by Thr). The mutations at positions 2, 5, 14, 65 and 72, were highly conservative in terms of hydropathy and structure (Dayhoff et al. 1978). Consequently, the replacements which are likely to be involved in the control of the level of necrotic activity with respect to the protective role of elicitins were restricted to only three positions (13, 61 and 94). Nevertheless, only position 13 is the site of a replacement resulting in a major change in hydrophobicity. It is worth noticing that this mutation is correlated with those occurring at positions 2, 5, 14, 72 and 94. The reasons might be an early gene divergence or a structural hindrance.

The comparison of the CD spectra shows that the secondary structure of elicitins is not largely affected by the point mutations. Taking into account the CD data in order to modulate the decision constants in the prediction of secondary structures leads to consistent results. These predictions were nevertheless quite different from those already published (Ricci et al. 1989), obtained with default decision constants used in the absence of CD measurements. Elicitins appear now to be ordered proteins, exhibiting little or no β-structure, but having an α-helix for half of the molecule, while β-turns and aperiodic structure constitute equally the rest of the protein. The alterations in the optimized predicted secondary structures that correlate with the differences in toxicity

only account for less than 10% of the residues. These residues are found in the same region as those revealed by the hydropathy profiles. Consequently, the interchange of a hydrophilic residue (Lys) at position 13 to a hydrophobic one (Val) appears to be the major event which lowers the necrotic activity.

Comparisons of the complete sequences, the amino-acid compositions, the isoelectric points, the hydropathy indices and the predicted secondary structures of the four elicitins lead us to classify these molecules into two groups: the class α corresponds to acidic molecules with a valine residue at position 13, while the class β is characterized by a lysine at that position and a basic isoelectric point. These two classes fit the distinction based on the necrotic properties: β elicitins are more toxic than α ones.

The elicitation of necrosis involves the migration of the elicitin in the plant indicating that there is no difference in migration between cryptogein and capsicein (data not shown). The difference in necrotic properties of classes α and β is therefore to be found in structural features involved in the interaction of elicitins with the target cells in the lamina of the leaf. The structural differences which are correlated with the necrotic activities probably involve residues bulging at the surface of the elicitin molecule, whereas the global structure is not altered, as demonstrated through CD. This supports the hypothesis that elicitins have an active site which is close to the variable domains and is responsible for the induction of necrosis. Position 13 appears to be a putative site for such an activity. Such information will be essential for engineering elicitin-like molecules devoid of their necrotic properties, for protection against *Phytophthora*. Nevertheless, the mechanisms involved in the elicitation of plant defence reactions are still not understood and need further investigation.

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