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A novel elicitin necrotic site revealed by α-cinnamomin sequence and site-directed mutagenesis

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Abstract

Elicitins are 10 kDa proteins secreted by *Phytophthora* fungi, that elicit resistance against certain plant pathogens. Various natural molecules, mutated recombinant elicitins and synthetic peptides were previously shown to differentially induce in tobacco leaf necrosis and defence genes, activities borne by several sites which were identified. We report a novel necrosis-determining residue at position 25, revealed by the comparison of the necrotic activity and sequence of α-cinnamomin with those of other known elicitins. Using a modified recombinant β-cryptogein, expressed in *Pichia pastoris*, we show that the substitution of asparagine 25 by a serine leads to a significant enhancement of the necrotic activity.

Keywords: Amino acid sequence; Elicitin; Necrotic site; *Nicotiana tabacum*; *Phytophthora cinnamomi*; Site-directed mutagenesis

1. Introduction

Fungi of the genus *Phytophthora* are a major cause of crop devastation. In tobacco most *Phytophthora* species induce generalized defence responses accompanied by only limited tissue decomposition. The active agents are small proteins (98 amino acids), termed elicitins, which are abundantly secreted into the culture medium by most of the *Phytophthora* species, with the exception of *Phytophthora parasitica* var. *nicotianae* (Ricci et al., 1989; Nespoulous, Huet, & Pernollet, 1992; Pernollet, aSallantin, Sallé-Tourne, & Huet, 1993; Kamoun, Young, Glascock, & Tyler, 1993; Kamoun, Young, Förster, Coffey, & Tyler, 1994). These elicitor proteins induce remote leaf necrosis in infected tobacco plants and generate defence responses which include a hypersensitive-like reaction, leading to a systemic acquired resistance against infection by the major tobacco pathogen (i.e. *P. parasitica* var. *nicotianae*) (Ricci et al., 1989) and also bacterial plant pathogens (Kamoun et al., 1993). The severe, virulent, lethal pathogenicity of *P. parasitica* var. *nicotianae* on tobacco appears to be correlated to the absence of elicitin secretion, the host does not perceive the fungus and thus fails to initiate defence mechanisms. Transformation of tobacco with a gene encoding β-cryptogein produced an intracellular accumulation of elicitin in the plant before attack. This provided the necessary protein signal not produced by the *P. parasitica* var. *nicotianae* and resulted in resistance to the pathogen (Tepfer et al., 1998).

The complete sequences of 12 elicitins are known (Ricci et al., 1989; Huet & Pernollet, 1989, 1993; Nespoulous et al., 1992; Huet, Nespoulous, & Pernollet, 1992; Huet, Mansion, & Pernollet, 1993; Huet, Sallé-Tourne, & Pernollet, 1994; Huet, Le Caer, Nespoulous, & Pernollet, 1995), consist of 98 amino acids, and show >67% sequence identity. In β-cryptogein, three conserved disulfide bridges maintain a 3-D structure which comprises six α-helices and a peculiar motif, whose sequence is highly conserved among the elicitin family, composed of a short antiparallel two-stranded β-sheet and an Ω loop (Boissy et al., 1996; Fefeu, Bouaziz, Huet, Pernollet, & Guittet, 1997). Elicitins display a variable ability to induce necrosis and independently trigger defence responses, with β-(basic) elicitins producing more necrosis than α-(acidic) elicitins (Nespoulous et al., 1992; Pernollet et al., 1993; Huet et al., 1995). Possible necrotic-determining residues can be identified at positions 2, 13, 72 and 94. The nature of the side-chain of residue 13 is highly correlated to necrotic potential, as demonstrated by the results of a mutagenesis study of this position in a

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synthetic β-cryptogein gene (O'Donohue et al., 1995). Other sites responsible for the residual necrosis induction of the α-elicitins have been determined together with those responsible for inducing plant defences using a series of synthetic peptides and tobacco plants transformed with a reporter gene controlled by defence gene promoters (Perez, Huet, Nespoulous, & Pernollet, 1997).

The comparison of necrosis induction by various elicitin isoforms has previously shown that the acidic and basic isoforms of cinnamomin secreted by *Phytophthora cinnamomi* are the most necrogenic in their respective classes (Pernollet et al., 1993). In this paper, we report the complete sequence of α-cinnamomin and reveal a novel key residue by comparison to the β-cinnamomin sequence (Huet & Pernollet, 1989) and those of other elicitins. Site-directed mutagenesis of the synthetic β-cryptogein gene expressed in *Pichia pastoris* (O'Donohue et al., 1996) provide further evidence for the prominent role of this residue in necrosis induction.

2. Results

2.1. Amino acid sequencing of α-cinnamomin

The N-terminal sequence was determined with 4-vinylpyridine reduced and alkylated α-cinnamomin. Overlapping peptides (Fig. 1) were obtained either after digestion with CNBr or cleavage of the performic acid oxidized protein with trypsin. With an initial yield of 70% and a repetitive sequencing yield of 93%, the N-terminal end was sequenced up to Ala 38 and was found to be overlapping with the CNBr peptide B1. The tryptic peptide Ox1 allowed the alignment of the peptides B1, B2 and B3. The latter overlapped with the C-terminal tryptic peptide Ox2. The good agreement between the IS-MS measured *Mr* (10,206.5±0.9) and that calculated from the amino acid sequence assuming three disulfide bridges as in other elicitins (10,206.7) validates the sequence of α-cinnamomin. It comprises 98 amino acid residues and is devoid of side chain modification, like other *Phytophthora* elicitins and in contrast to *Pythium* elicitin-like proteins (Huet et al., 1995). The far UV circular dichroism (CD) spectrum of α-cinnamomin (not shown) was quite comparable to those of natural (Nespoulous et al., 1992; Huet et al., 1992) and recombinant (O'Donohue et al., 1996) elicitins.

2.2. Sequence homology of elicitins

Figure 2 illustrates the alignment of the α-cinnamomin sequence with those of mature elicitins secreted by *Phytophthora* species and the two recombinant proteins used in this study. The natural sequences are ordered from top to bottom according to increasing necrotic power to tobacco detached leaves (Pernollet et al., 1993). Boxes indicate residues involved in elicitin biological activities, thick lines correspond to the sites determined by sequence comparison and site-directed mutagenesis, thin lines those identified by synthetic peptides (Perez et al., 1997). The strict homology between both cinnamomins (84.7%) is comparable to the sequence homologies of other elicitin isoforms belonging to a same species (i.e. 92% for *P. drechsleri* elicitins (Huet et al., 1992) and 87% for *P. megasperma megasperma* elicitins (Huet & Pernollet, 1993). The differences between α- and β-cinnamomins are essentially those which are commonly found between α- and β-elicitins. Six heterologous residues were located at positions 2, 13, 28, 72, 93 and 94, which belong to the 11 sites already identified to be involved in the elicitin biological activities. Comparison of cinnamomins to the other 11 natural *Phytophthora* elicitin sequences showed complete homology and no deletion was necessary for alignment. The match between this set of elicitins was 67.3% and showed large consensus regions, emphasized in Fig. 2 by a solid line which separates acidic from basic elicitins. The α-cinnamomin sequence exhibited only one novel feature, distinguishing cinnamomins from other elicitins. This difference, indicated by bold circles in Fig. 2, corresponds to the replacement at position 25 of an Asn by a Ser.

2.3. Characterization of recombinant mutated elicitins

In order to confirm the peculiar role of the residue 25, we decided to change β-cryptogein Asn 25 into Ser to test

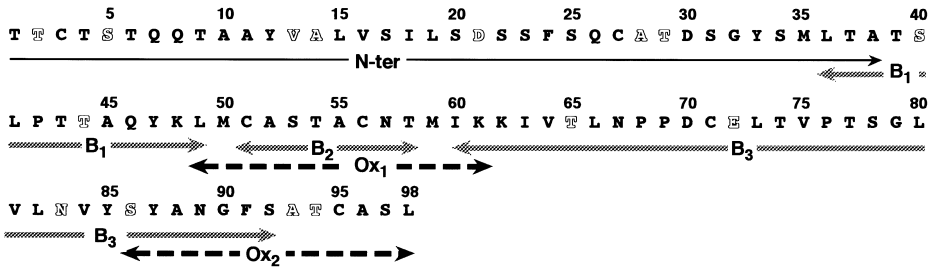

Fig. 1. Alignment of α-cinnamomin peptides. N-ter is N-terminus of reduced and alkylated α-cinnamomin, B_1, B_2 and B_3 are CNBr-cleaved peptides, Ox_1 and Ox_2 are tryptic peptides obtained after digestion of the oxidized protein with performic acid. Outlined residues indicate those different from β-cinnamomin.

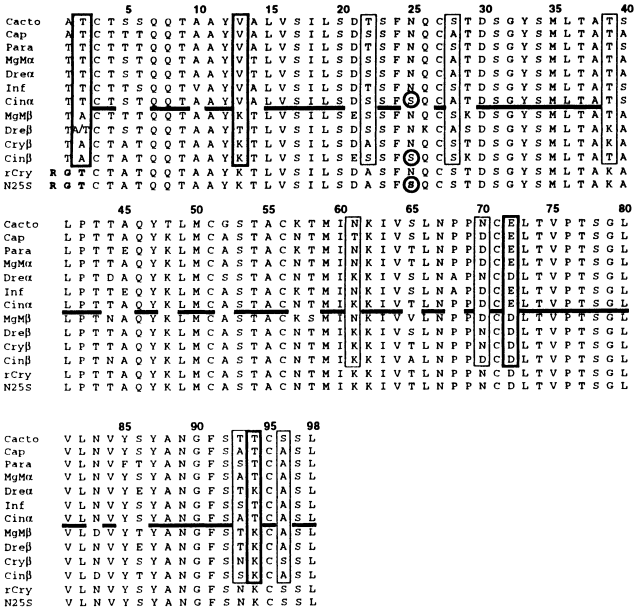

Fig. 2. Sequence comparison of natural and recombinant *Phytophthora* elicitins. Cacto, *Phytophthora cactorum* elicitin (Huet et al., 1993); Cap, capsicein, *Phytophthora capsici* elicitin (Ricci et al., 1989); Cinα and β, *P. cinnamomi* acidic and basic elicitins, respectively (this work and Huet & Pernollet, 1989); Cryβ, β-cryptogein, *Phytophthora cryptogea* basic elicitin (Ricci et al., 1989); Dreα and β, *Phytophthora drechsleri* acidic and basic elicitins, respectively (Huet et al., 1992); Inf, *Phytophthora infestans* elicitin (Huet et al., 1994); MgMα and β, *Phytophthora megasperma megasperma* acidic and basic elicitins, respectively (Huet & Pernollet, 1993); Para, parasiticein, *P. parasitica var parasitica* elicitin (Nespoulous et al., 1992); rCry, recombinant β-cryptogein (O'Donohue et al., 1996); N25S, rCry mutated at position 25 (this work). The natural sequences are ordered from top to bottom according to increasing necrotic power to tobacco detached leaves (Pernollet et al., 1993). Circles indicate the residues typical of *P. cinnamomi* elicitins. Bold characters show the residues of the recombinant elicitins which have been modified compared to β-cryptogein. The regions of consensus are emphasized with a continuous line which separates acidic from basic elicitins. Boxes indicate residues involved in elicitin biological activities, thick lines correspond to the sites determined by sequence comparison and site-directed mutagenesis, thin lines those identified by synthetic peptides and activation of defence genes.

the influence of this sole mutation. The production of large quantities of highly pure recombinant natural (rCry) or mutated (N25S) β-cryptogeins was achieved using the *P. pastoris* expression system as described by (O'Donohue et al., 1996). The N-termini of these molecules exhibit some minor sequence differences with respect to wild-type β-cryptogein, since they were designed to optimize the signal peptide removal (i.e. Thr 1 is replaced by Gly and Ala 2 by Thr, and an additional Arg constitutes the N-terminal amino-acid) as reported in Fig. 2. Both the N-terminal sequences and the *Mr* of the purified recombinant proteins were verified prior to biological assays: rCry exhibited a measured *Mr* of 10,471.3 ± 1.2 (calculated 10,471.1) and N25S a *Mr* of 10,444.4 ± 1.0 (calculated 10,444.0).

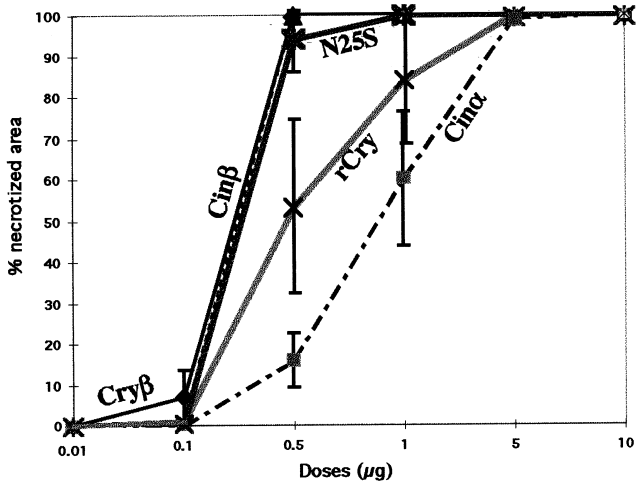

Fig. 3. Comparison of necrosis induction on tobacco detached leaves by elicitins. Cryβ (– ◆ –), β-cryptogein, *Phytophthora cryptogea* basic elicitin; Cinα (– ■ –) and Cinβ (– ▲ –), acidic and basic *Phytophthora cinnamomi* elicitins, respectively; rCry (– × –), recombinant β-cryptogein and N25S (– ∗ –), recombinant mutated β-cryptogein. The vertical ordinate indicates the mean proportion of necrotized area measured on five detached leaves, standard deviations are indicated when different from zero. Detached leaves that were kept in nutrient solution at room temperature in the dark for 48 h.

2.4. Necrotic properties on tobacco leaves

Figure 3 shows the dose–response curves obtained with α- and β-cinnamomins, β-cryptogein and the recombinant proteins. The endpoint responses (0 and 100% necrotized area) were identical from one experiment to another, whereas the transition responses were subject to some variability (standard deviations indicated in Fig. 3). Cinβ and Cryβ were found to be the most necrotic molecules, whereas Cinα was the less active one. These values are in good agreement with the previous observations (Pernollet et al., 1993). The recombinant protein rCry was found to induce necroses in a range close to that of Cinα, while the mutated N25S recombinant elicitin was nearly as necrotic as Cinβ.

3. Discussion

Compared to the other *Phytophthora* elicitins, α-cinnamomin exhibits comparable size, amino acid composition, absence of side-chain modification and global conformation, as revealed by circular dichroism. The higher necrosis induction of α- and β-cinnamomins, compared to acidic and basic elicitins, respectively (Pernollet et al., 1993), appears to be correlated with the presence of serine instead of asparagine at position 25. This potential, novel necrotic-determining residue was directly investigated by site directed mutagenesis of a recombinant β-cryptogein produced by the *P. pastoris* system.

The determination of the necrotic potential of rCRY

revealed that the presence of an extra Arg at the N-terminus of the β-cryptogein recombinant protein significantly reduces its necrotic power, compared to that of the wild-type Cryβ, to almost the level of Cinα. Examination of the 3-D structure of β-cryptogein (Boissy et al., 1996; Fefeu et al., 1997) shows that seven of the 13 residues (including Arg 0) involved in the biological activities are grouped in two clusters at the borders of a depression centered around Tyr 87. One of these sites involves the active residues 70 and 72 (Perez et al., 1997), which are located close to the extra Arg 0 (Fig. 4a) whose positive charge considerably alters the electric potential of this region, which in Cryβ is principally due to Asp 72. This modification probably explains the loss of necrotic activity associated with this extra arginine, but the presence of a threonine at position 2 instead of alanine might also contribute to the necrotic activity reduction (Perez et al., 1997).

The replacement of Asn 25 by Ser in rCRY, thus imitating the natural cinnamomin sequence context, significantly restored the necrotic activity of this protein to a level close to that of β-cryptogein and β-cinnamomin. Fig. 4 shows that the residue 25 is located in a solvent-exposed position on the second α-helix close to Ala 22 and Ser 28, amino acids previously shown to be of importance for biological activity (Perez et al., 1997). Our present data indicate a crucial, necrotic-determining role for these three exposed residues. Interestingly, these residues are located on the same face of the elicitin as residue 13, a key amino acid for necrosis induction (O'Donohue et al., 1995; Perez et al., 1997). It is therefore very likely that residues 22, 25 and 28 are involved in a molecular interaction which also involves residue 13.

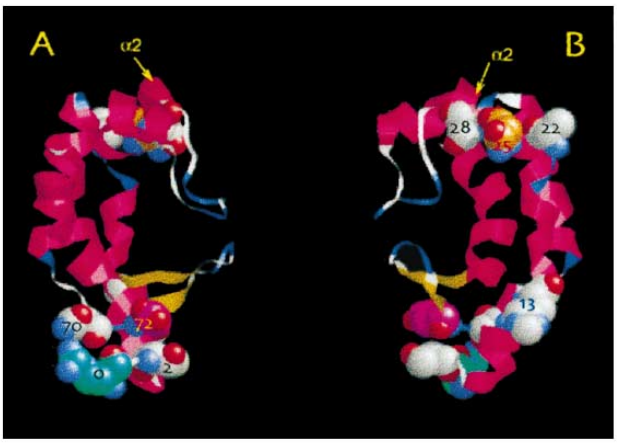

Fig. 4. Location of key residues in the 3-D structure of β-cryptogein. Protein Data Bank identification of β-cryptogein coordinates: 1BEO (Boissy et al., 1996). Substantial aminoacids, numbered according to Fig. 2, are shown in space-filling model, the rest of the molecule as strand. The second α-helix is indicated by α2. (a) The concave face showing the crevace centered around Tyr87, the additional N-terminal Arg residue (carbon atoms colored in cyan) and the active residues 2, 70 and 72 (carbon atoms colored in magenta). (b) The opposite convex face showing the residues 13, 22, 25 (carbon atoms colored in yellow) and 28.

The optimization of elicitin-induced resistance in plants expressing an elicitin-encoding gene would necessitate the simultaneous augmentation of the elicitor potency of the transfected gene product and the negation of its high necrotic power. However, to attain this attractive biotechnological goal, all of the amino acids involved in both elicitin activities need to be accurately identified. These present results, along with previously published data on the necrotic-determining residues of elicitins, go a long way to meeting this requirement and the development of tailored-made elicitin genes for use in tobacco plant transformation can now be envisaged. These molecules will have an increased ability to elicit resistance against *Phytophthora* while lacking undesirable side effects such as necrosis development. Despite the numerous studies which have provided an experimental description of many of the cellular events that arise from elicitin activity, the comprehensive molecular basis of the host specificity of *Phytophthora* is not currently known and the identification of the biological functions of elicitins still remains to be established (Grant, Ebert, & Gayler, 1996). Our present results also contribute to the understanding of this novel family of proteins which comprises numerous related members distinct from authentic elicitins whose precise modes of action are still unknown (Baillieul, Fritig, & Kauffmann, 1996; Kamoun, Lindqvist, & Govers, 1997).

On a more general point of view, these results confirm several reports on structure–activity relationships studies of other fungal elicitors that have also proven that single changes in the amino acid sequence may significantly alter their biological activity, as shown for a peptide originating from a 42 kDa glycoprotein elicitor from *P. megasperma megasperma* (Nürnberger et al., 1994) and the AVR9 peptide of *Cladosporium fulvum* (Kooman-Gersmann, Voselgang, Hoogendijk, & De Wit, 1997). This supports the idea that fungal phytopathogens are being recognized by the plant in a receptor-like manner.

4. Experimental

4.1. Protein purification

Natural proteins (α- and β-cinnamomins and β-cryptogein) were purified according to the general elicitin procedure using the fungi isolates as described in (Pernollet et al., 1993). Recombinant proteins secreted by *P. pastoris* were purified as described by O'Donohue et al., 1996. The elicitin proteins were lyophilized and stored at −20°C before use. Their purity was checked using analytical RPLC, N-terminal sequencing and mass spectrometry. Both the N-terminal sequences and the *Mr* were verified before biological assays.

4.2. Protein sequencing

Fifty μg of α-cinnamomin were reduced with 2-mercaptoethanol and alkylated with 4-vinyl-pyridine according to Henschen, 1986. The reduced and alkylated protein was submitted to N-terminal Edman degradation and to peptide cleavage with CNBr (Huet et al., 1992). In addition, 50 μg of α-cinnamomin, previously oxidized with performic acid, were digested with sequencing grade modified trypsin (EC 3.4.21.4) obtained from Promega according to (Huet et al., 1993). Automated Edman degradation was performed using either an Applied Biosystems 475A sequencer and its on-line phenylthiohydantoin amino acid analyzer model 120A or a Perkin Elmer Procise 494-610A sequencer with reagents and methods of the manufacturers.

4.3. Protein characterization

Mr were determined by micro ion spray mass spectrometry (Sciex API100, Perkin-Elmer) using a positive mode from 300 to 3000 a.m.u. with 0.5 a.m.u. steps and a 1 ms dwell-time. The ion spray needle voltage was 6000 V and the orifice plate voltage 40 V, at a flow rate of 0.2 $\mu l \cdot min^{-1}$. Mass spectrometry data were analyzed with the Perkin Elmer Sciex Bio-Multi-View 1.2 software. The average *Mr* were calculated from the sequence using the Perkin Elmer Sciex Peptide Map 2.2 software. Circular dichroism spectra were recorded using a Mark V Jobin-Yvon (Longjumeau, France) dichrograph, as previously reported (O'Donohue et al., 1996).

4.4. Recombinant protein production and site directed mutagenesis

The production of recombinant elicitins was performed in *P. pastoris* according to (O'Donohue et al., 1996). The position 25 was mutated from Asn to Ser using a Transformer™ mutagenesis kit (Clontech), based on the unique site elimination system (Deng & Nickoloff, 1992) as previously described (O'Donohue et al., 1995). The mutagenic primer sequence (with the mutant serine anticodon TCG in italic) was: 5′p AGA GCA CTG *GCT* GAA AGA CGC GTC 3′. The selection primer was designed to direct the substitution of an *Afl* II recognition site by a *Nde* I site; the sequence of this primer (with the *Nde* I site in italic) was: 5′p TCA CAC CG*C ATA TG*G TGC ACT C 3′. Both proteins differ from the wild-type β-cryptogein at their N-terminus (RGT instead of TA).

4.5. Tests for necrotic activities on tobacco leaves

Purified natural and recombinant proteins were tested from approximately 60-day old greenhouse grown tobacco plants (*Nicotiana tabacum* cv *Xanthi* XHFD8) by petiole inoculation on detached leaves. Inoculations of 10 μl were applied to still expanding leaves with elicitin amounts of 10 ng, 100 ng, 500 ng, 1 μg and 10 μg diluted in pure water. Water was also inoculated as a negative control. All solution concentrations were spectrophotometrically checked by UV absorbance at 277 nm with the proper extinction coefficient (Nespoulous & Pernollet, 1994) before they were inoculated. Once the elicitin solution drop had been taken up by the petiole, usually within 30 s, the petiole was dipped into a nutrient solution adapted from (Baudet et al., 1986). Treated leaves were incubated at room temp in the dark to allow the necroses to develop. Five replicates of each treatment were included in each experiment and all elicitin molecules were assayed simultaneously to reduce variability. Symptoms reached their maximum extent after 2 days. The relative necrotic index of an isoform was defined as the sum of the normalized necrotic area, equal to 1 when a leaf was totally necrotized and zero for no necrosis, obtained with doses of 10 ng, 100 ng, 1 and 10 μg.

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